IMMUNOLOGICAL AND EPIDEMIOLOGICAL INVESTIGATIONS INTO AVIAN MALARIA IN THE AFRICAN PENGUIN DURING REHABILITATION AND IN BREEDING COLONIES

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Declaration

I, the undersigned, hereby declare that work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Signature

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Date

Opsomming

Die Afrika Pikkewyn kom langs die suid-oostelike en suid-westelike kus van Suid Afrika en Namibië voor. In die afgelope eeu het hierdie spesie ‘n geweldige afname in populasie getalle ondervind. Dit was hoofsaaklik die gevolg van die versameling van guano en pikkewyneiers in die eerste helfte van die 19de eeu en oliebesoedeling in die tweede helfde van die 19de eeu. Die “South African Foundation for Conservation of Coastal Birds” (SANCCOB) is ‘n seevoëlreddings- en rehabilitasiesentrum vir siek, beseerde en ge-oliede pikkewyne. Dit word geskat dat die Afrika Pikkewyn populasie met ‘n verdere 19% sou afgeneem het as dit nie vir die rehabilitasie by die SANCCOB sentrum was nie. Hierdie sentrum het egter aansienlike vrektes in die somer as gevolg van voëlmalaria, wat sodoende die effektiwiteit van die rehabilitasie verlaag. In ‘n poging om die rol van immuniteit teen malaria te bepaal is ‘n “enzyme-linked immunosorbent assay” (ELISA) ontwikkel vir die bepaling van antiliggaam vlakke teen malaria. Hierdie ELISA is gebruik vir die bepaling van die anti-*Plasmodium* antiliggaam vlakke van die pikkewyne by aankoms en ten tye van rehabilitasie by SANCCOB vanaf Oktober 2001 to Januarie 2003.

Die doel van hierdie studie was eerstens om hierdie ELISA bepalings voort te sit om sodoende antiliggaam vlakke teen malaria oor twee kalender jare te kan evalueer. Hierdie ondersoek was gekombineer met ‘n polimerase ketting reaksie (PCR) metode, wat enige *Plasmodium* spesie in pikkewynserum sou kon opspoor. Hierdie twee metodes is ook gebruik vir ondersoeke in sommige broeikolonies, met die doel om te bepaal watter rol voëlmalaria in die oorlewing van die Afrika pikkewyn in die natuur speel.

Resultate het getoon dat olie nie die vermoë van die pikkewyn beïnvloed om anti-*Plasmodium* antiliggame te vervaardig nie en dat malaria infeksie hoofsaaklik deur muskiete veroosaak word en nie deur heruitbraak van ‘n bestaande infeksie nie. Dit dui egter daarop dat pikkewyne blootgestel word aan voëlmalaria by die SANCCOB sentrum. Daar is ook gevind dat ‘n groot aantal pikkewyne met malaria infeksies by die sentrum opgedaag het wat dui op die voorkoms van malaria in die broeikolonies. Ondersoeke in die broeikolonies het ‘n besonder hoë voorkoms van malaria onthul. Geen vrektes of siek pikkewyne is in die broeikolonies waargeneem nie, wat moontlik kan beteken dat pikkewyne by SANCCOB met ‘n ander tipe malaria geïnfekteer word as in die broeikolonies.

Summary

The African penguin, which occurs along the south-eastern and south-western shores of South-Africa and Namibia, has experienced a severe reduction in population numbers due to guano and egg collection in the first half of the 19th century, and oil pollution in the second half of the 19th century as a result of oil tankers rounding the Cape of Good Hope. The population would have been reduced by a further 19% had it not been for the rehabilitation of penguins at the South African National Council for the Conservation of Coastal Birds (SANCCOB) facility. Although this has been very successful, mortalities as a result of avian malaria infection have considerably reduced the efficiency of rehabilitation. In an effort to assess the role of immunity against malaria in combating the disease, an enzyme-linked immunosorbent assay (ELISA) for the detection of antibody levels to avian malaria was developed. The ELISA was used to detect antibody levels to avian malaria of penguins on entry and during rehabilitation from October 2001 to January 2003.

The aim of this study was to continue the determination of antibody levels to avian malaria of penguins entering the SANCCOB facility, in order to allow an evaluation of the antibody levels to avian malaria for two full calendar years. This investigation was combined with a polymerase chain reaction (PCR)-based method, capable of detecting any *Plasmodium* species in penguin serum. These two methods were also used to investigate avian malaria in several breeding colonies in order to assess the role avian malaria may play in the survival of the African penguin in the wild.

Results indicated that the ability of penguins to produce anti-*Plasmodium* antibodies was not influenced by oiling and that infection with malaria was not due to recrudescence but rather due to infection via mosquitoes. This indicated a possible role of the SANCCOB facility in exposing the penguins to avian malaria. However a large number of penguins arrived at the facility previously infected with malaria, indicating that malaria was present in the breeding colonies. Investigations in the breeding colonies revealed extremely high avian malaria prevalence even though no sick birds or mortalities were observed. This raised the question whether different types of malaria are responsible for infection in the SANCCOB facility and breeding colonies.

Abbreviations

A	Adenine
ABTS	Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
B	Stony Point, Betty's Bay
BI	Bird Island, Algoa Bay
BSA	Bovine serum albumin
C	Cytosine
CITES	Convention on Trade in Endangered Species
CS	Circumsporozoite
D	Dassen Island
DMF	N,N-dimethyl formamide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylene diamine tetra-acetic acid di-sodium salt
ELISA	Enzyme-linked immunosorbent assay
G	Guanine
GLM	General Linear Models
h	hour
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITS	Internal transcribed spacer
IUCN	The World Conservation Union
Kb	Kilobases

LSD	Least significant difference
LSU	Large subunit
LYMPH	Relative lymphocytosis
Mb	Megabases
Min	Minutes
NANP	Asparagine-Alanine-Asparagine-Proline
P	Probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
R	Robben Island
RIA	Radioimmunoassay
RBCs	Red blood cells
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SANCCOB	The South African National Foundation for the Conservation of Coastal Birds
SAS	Statistical Analysis System
SSU	Small subunit
T	Thymine
Taq	*Thermus aquaticus*
TE	Tris-EDTA
UCT	University of Cape Town
WBC	White blood cell

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CHAPTER 1

INTRODUCTION

The African Penguin, *Spheniscus demersus,* occur along the south-eastern and south-western shores of South-Africa and Namibia and is the only penguin species that breeds in Africa. Unfortunately this species has experienced a severe reduction in population numbers. The present population is about 10% of what it was at the start of the 20th century when it was an estimated 2 million. Currently the African Penguin is classified as "Vulnerable" in terms of the South African Red Data Book for birds, as well as the IUCN threatened species categories. Initial decline in numbers was due to direct exploitation by humans: hunting, guano scraping and egg collecting. Today one of the most important immediate threats facing the African Penguin is oil pollution.

It is estimated that the African Penguin population would have been reduced by a further 19% had it not been for the rehabilitation of penguins at the South African National Council for the Conservation of Coastal Birds (SANCCOB) facility in Milnerton, Cape Town (Ryan, 2003). SANCCOB has played a vital role in the rehabilitation of especially oiled, but also sick and injured penguins. Rehabilitated birds have been shown to return to their former colonies and continue to breed successfully (Wolfaardt and Nel, 2003). However, although the rehabilitation of penguins has been very successful, mortalities during the summer as a result of avian malaria infection have reduced the efficiency of rehabilitation efforts considerably. The recent (winter of 2000) oil spill of the ship "Treasure" some 100 km north of Cape Town, again showed how exposed this penguin species really is. Some 22 000 penguins were oiled, of which subsequently 95% were successfully rehabilitated thanks to the efforts of SANCCOB and the International Foundation of Animal Welfare. Had this oil spill occurred in summer, mortalities as high as 50-70%, due to avian malaria, may have been expected, as these are the mortalities routinely incurred during summer at the SANCCOB facility. The possibility that avian malaria may be contracted at the SANCCOB facility and thereby introduced into the already endangered wild populations is also a major concern.

In order to improve survival rates in the SANCCOB facility, a project was launched to establish the immunity of penguins to avian malaria upon entry and during rehabilitation. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibody levels to avian malaria was developed. This assay was used to detect anti-*Plasmodium* antibodies in African Penguins upon entry into the facility and during rehabilitation from October 2001 to December 2002, with a view to increasing the survival rate in the facility (Botes, 2004).

The aim of this study was to continue the determination of antibody levels to avian malaria of penguins entering the SANCCOB facility, allowing an evaluation of the antibody levels to avian malaria for two full calendar years. This included determining whether the ability of penguins to produce an anti-*Plasmodium* antibody response influences their survival rate; whether oiling influences the penguins' ability to produce an anti-*Plasmodium* immune response and whether penguins become infected at the facility or suffer from parasite recrudescence.

Infection of penguins during rehabilitation was also investigated using a specific polymerase chain reaction (PCR) assay for the detection of *Plasmodium* infections. The PCR results were used to determine whether African Penguins were infected with malaria prior to their arrival or during rehabilitation at the SANCCOB facility. PCR analysis included samples taken from Greywing Francolins caught in close proximity to SANCCOB in order to assess if they could serve as a possible avian malaria reservoir. SANCCOB is situated next to a large shallow freshwater lake (Rietvlei) with abundant bird life (possible malaria reservoirs) and culicine mosquitoes (vectors). If the position of SANCCOB places the penguins at greater risk to malaria infections, the facility may have to be moved to overcome this problem.

Lastly, the study was expanded to include penguins from several land and island colonies to assess the role avian malaria may play in the survival of the African Penguin in the wild and to evaluate the risk of releasing possibly infected rehabilitated penguins into the wild. Both ELISA and PCR analysis were used in this study.

An overview on the African Penguin is given in Chapter 2 and involves its distribution, conservation status and rehabilitation at SANCCOB. Chapter 3 focusses on malaria and in particular avian malaria in the African Penguin. The molecular and immunological techniques used in this study are discussed in detail in Chapter 4.

Results obtained from the penguins at SANCCOB are presented in Chapter 5 while the results obtained from penguins in the breeding colonies are presented in Chapter 6. A final conclusion and future perspectives are given in Chapter 7. The literature cited is listed at the end of the thesis followed by an Appendix containing the data used for statistical analysis of the results in Chapter 5 and Chapter 6.

CHAPTER 2

THE AFRICAN PENGUIN

2.1. Introduction

Penguins are a distinctive group of flightless, pelagic seabirds belonging to the family Spheniscidae. Worldwide there are only 17 species of penguin, all of which breed in the Southern hemisphere. The largest concentration of species occurs in cold temperate, sub-polar and polar waters. Almost every Antarctic and Sub-Antarctic island has more than one species and large breeding concentrations occur at localities along the coast of the Antarctic Peninsula and the Antarctic continent. Penguins also breed on mainland coasts and off-lying islands of southern Australia and New Zealand, on islands off the coast of southern and southwestern Africa, in Patagonia and the Magellanes region, and along the coast of Chile and Peru. The northernmost penguins live close to the Equator at the Galapagos Islands (Stonehouse 1975).

The African Penguin, *Spheniscus demersus*, is the only penguin species that breeds in Africa. African Penguins are also known by the names Jackass penguin and blackfooted penguin. The name “jackass penguin” is derived from the braying, donkey-like call of territorial males (Randall 1989). The penguin species closest related to the African Penguin is found along the coast of southern South America and are called the Humboldt Penguin *S. humboldti* and the Magellanic Penguin, *S. magellanicus*. These species are very similar in size, appearance and behaviour to the African Penguin. It’s only other close relative, the Galapagos Penguin, *S. mendiculus*, is found only on the Galapagos Islands and is the world’s most tropical penguin (Hockey 2001).

2.2. Distribution

The African Penguin occurs along the southeastern and southwestern shores of South Africa and Namibia, breeding on scattered islands along these coasts. Its distribution coincides roughly with the cool, northward-flowing and nutrient rich Benguela Current. The African Penguin’s breeding range extends from Hollamsbird Island, off central Namibia, to Bird Island in Algoa Bay, even though non-breeding birds often disperse as

far as KwaZulu-Natal and southern Angola. African Penguins currently breed at 27 colonies, eight islands and one mainland site along the coast of southern Namibia, 10 islands and two mainland sites along the coast of the Western Cape Province (South Africa), and six islands in Algoa Bay (Eastern Cape Province, South Africa). Breeding no longer occurs at 10 localities where it formerly occurred or has been suspected to occur. Nevertheless, 77% of the population currently breed on only four islands and

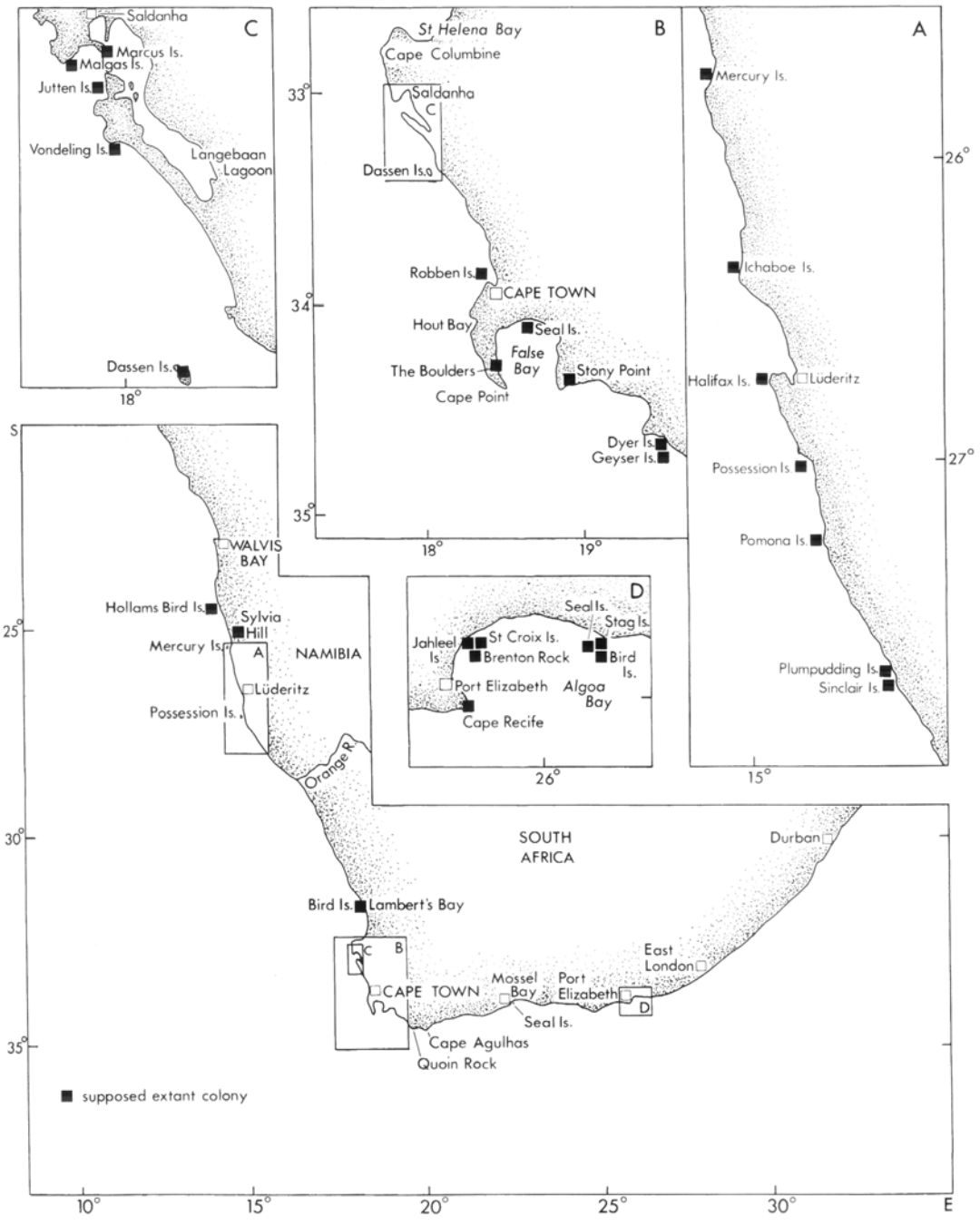


Figure 2.1: Extant breeding colonies of African Penguins (Randall 1989).

more than 80% of the population breed in only two small and distinct geographic areas. One of these areas are the islands between Saldanha Bay and Cape Town where more than 40% of African Penguins breed while almost another 40% of African Penguins breed within Algoa Bay (Randall 1989, Nel *et al*., 2003, Bingham 2004, Wolfaardt 2004).

2.3. Conservation status

The present population of the African Penguin is about 10% of what it was at the start of the 20th century when it was an estimated two million (figure 2.1). In the 1950s the population had declined to less than 300,000 adults and by the late 1970s numbers had fallen to only 220,000. By the late 1980s, the population was down to 194,000 adults and in the early 1990s only 179,000 adults remained. By the late 1990s the population had recovered slightly, and in 1999 there were an estimated 224,000 birds (Hockey 2001). The African Penguin is classified as Vulnerable in terms of the South African Red Data Book for birds (Barnes 2000) and the IUCN threatened species categories (Birdlife International 2000). It is also listed in Appendix II of CITES and the Bonn Convention for conservation of migratory species (Underhill 1996).

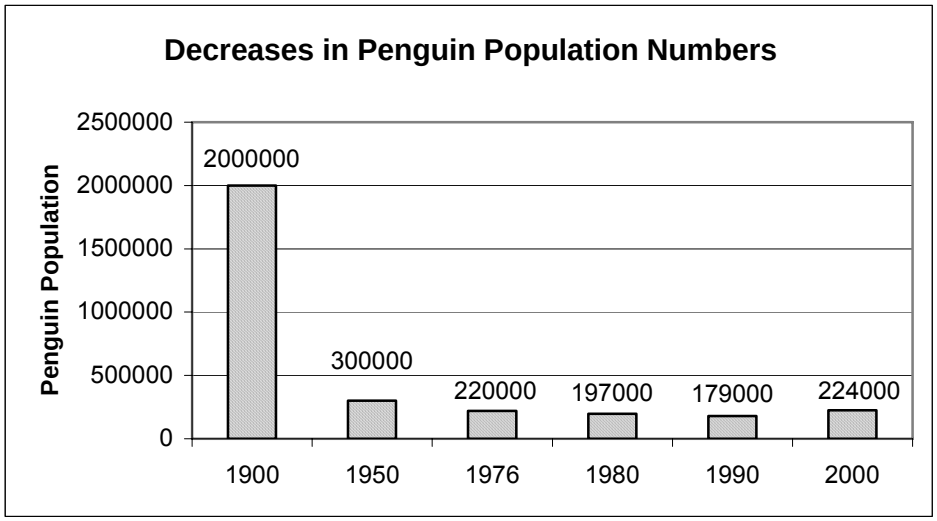


Figure 2.1: Graph showing the decrease in the African Penguin population.

2.4. Past and present threats

A variety of factors have been cited as contributing to the decline in the penguin population and different factors have influenced population size at different times.

2.4.1. Penguin collection

Initially, the decline in the African Penguin populations was driven by direct exploitation by humans. Collection of penguins for provisions on ships began with the arrival of the first European explorers in the late 15th century. Although the flesh was not very pleasant to eat, the sailors still salted the penguins for provision and the eggs were considered a delicacy. The carcasses of birds were rendered down both for fat and as fuel for ships' boilers. Exploitation became intense with the arrival of the first settlers at the Cape in 1652 and almost led to the extinction of the population on nearby Robben Island (Rand 1949).

2.4.2. Guano collection

In the early 1840s the South American guano rush spread to Africa. Guano can be transformed into a nutrient-rich agriculture fertilizer. Guano exploitation led to the removal of the soft guano substrate required by the penguins for burrowing and successful breeding. Literally mountains of accumulated guano were stripped, leaving bare rock behind and dramatically altering the penguins' breeding environment. At Namibia's Ichaboe Island the guano cap of 23 m was removed. With the guano removed, the penguins were forced to nest in the open on the rocky island surface, where they were exposed to the elements and predators (Hockey 2001).

2.4.3. Egg collection

Egg harvest which began with the arrival of the first ships, was conducted on an ever-increasing scale until the 1930s. Initially eggs were collected as a cheap source of protein and over time with increasing scarcity, they became a luxury food. Dassen Island is one of several islands on which penguin eggs were collected for commercial purposes. It is estimated that the penguin population on the island was 1 400 000 at the end of the 19th Century. A phenomenal 13 million eggs were collected over the 30-year

period from 1900 to 1930, and as recent as 1956 about 126 800 eggs were collected. Today fewer than 17 000 breeding pairs remain on Dassen Island. Egg harvesting decreased during the 1960s and was suspended in 1969 (Randall 1989, Hockey 2001).

2.4.4. Competition for food

The above factors have now largely ceased, and the major current threats include competition with commercial fisheries for pelagic fish prey, and oil pollution. Investigations showed that prior to the peak of commercial fish exploitation, the penguin, gannet and Cape cormorant fed on the commercially important fish stocks of anchovy, *Engraulis capensis*, pilchard, *Sardinops ocelatta,* and maasbanker, *Trachurus trachurus*. The industries were initially based on exploitation of pilchard and maasbankers, but overexploitation resulted in massive decreases in the catches of these species in the 1960s. This shifted the attention to the smaller anchovy. Declines such as these affects the ecology of other marine organisms. The gannet and Cape cormorant are capable of ranging over a wide area in search of food, while the flightlessness of the penguin limits its feeding range and penguins must be able to rely on a highly predictable temporal and spatial distribution pattern of prey (Westphal & Rowan 1970, Frost *et al*., 1976). Consequently, the depletion of food supplies also led to a decline in the African Penguin population (Crawford *et al*., 1990).

2.4.5. Oil pollution

Oil pollution has had a major impact on African Penguins. Even though catastrophic oil spills occur irregularly, there is persistent, chronic oiling. The coast of southern Africa lies alongside one of the major shipping routes and is therefore at high risk of oil pollution. The incidence of oil pollution at the coast of southern Africa increased dramatically after the closure of the Suez Canal in 1967 (Westphal & Rowan 1970).

The African Penguin is concentrated in colonies and a single oil spill can have devastating effects. African Penguins are particularly susceptible to marine oil pollution, because they are flightless and spend most of their time at sea or near the surface of the ocean. Therefore, if oil covers their feeding grounds or landing areas at the breeding colony, the penguins will inevitably become oiled.

External oiling disrupts feather structure, causing matting of feathers and eye irritation. The African Penguin therefore loses its insulation and cannot survive in cold water. Even at temperatures of 20°C the penguin can become hypothermic and die. Alternatively, if the penguin remains on land too long, it is at risk of dehydration and starvation (Culik *et al*., 1991, Hockey 2001, Nel *et al*., 2003). Oiled penguins can also swallow the toxic pollutant through feather preening, drinking, consumption of contaminated food, and fumes from evaporating oil.

Ingestion of oil is seldom lethal, but can cause various debilitating sub-lethal effects that can promote mortality from other causes, including starvation and disease. These effects include inflammation and haemorrhaging of the digestive tract, red blood cell damage, hormonal imbalance, organ damage, inhibited reproduction, retarded growth in young, and abnormal parental behaviour (Miller *et al*., 1978, Alberts 1990). The toxic effect on red blood cells is a direct toxic effect either of compounds present in oil or of these compounds after metabolic conversion. The toxic mechanism involves destructive oxidation of red cell membranes and proteins. Anaemia has a significant metabolic cost to an animal and requires an increase in basal metabolism to maintain normal function. The mechanism of toxicity of the other above named effects is not yet known.

Bird embryos are also very sensitive to petroleum. The shell surface of the egg can be polluted by contaminated nest material and oiled plumage. Small quantities of certain types of oil are adequate to cause mortality, mostly during early stages of incubation. The pathology of embryos from oil-contaminated eggs revealed liver necrosis, generalized edema, degenerative changes in kidney, and enlargement of heart, spleen and liver (Leighton *et al*., 1983, Leighton 1990).

2.4.6. Natural threats

Other threats include competition with Cape Fur Seals, *Arctocephalus pusillus,* for space at breeding colonies and for food resources, whom together with the Great White Shark, *Carcharion carcharias,* and the Killer Whale, *Orcinus orca,* are natural predators of the penguin in the water. African Penguins also face predation of eggs and chicks by avian predators such as Kelp Gulls and Sacred Ibises, while natural terrestrial

predators, such as mongoose, genets, feral cats and leopards are present at the mainland colonies (Hockey 2001).

2.5. Rehabilitation at SANCCOB

As populations of wild animals decrease in size they become more vulnerable to stochastic events that can trigger further declines (Frankam *et al*. 2002). The vulnerability of African Penguins is increased further by its concentration within two relatively small geographic areas, both located close to major shipping ports. Consequently, catastrophic events, in the form of large-scale oil spills affecting thousands of birds, have now become one of the most immediate threats facing African Penguins.

A series of oil spills on the South African coast in the late 1960s led Mrs Althea Westphal to establish SANCCOB (the South African National Foundation for the Conservation of Coastal Birds). SANCCOB was originally based at Mrs Westphal's home but it grew to become an organisation with its own dedicated cleaning station situated at Milnerton (Bloubergrant) in Cape Town and an international leader in coastal bird rehabilitation. This volunteer organisation, although caring for sick injured or polluted seabirds, is dedicated largely to the de-oiling of penguins (Coultas and Cridland, 2004). A total of 47 000 oiled penguins have been admitted to SANCCOB over the past three decades, at an average of 1 500 birds per year. The incidence of oiling has varied greatly over this time and 77% of birds have been oiled between 1991 and 2000 (Nel *et al*., 2003).

Over the past three decades about 74% of sick and oiled penguins that have been admitted to SANCCOB were released back into the wild in a healthy condition. Release rates have improved greatly over the years because rehabilitation procedures have constantly been improved and refined. Furthermore, the 10 oldest African Penguins in the wild include four that had been treated by SANCCOB. A recent study on the post-release survival of rehabilitated African Penguins (Whittington 2003) tested the effectiveness of SANCCOB's rehabilitation of oiled African Penguins, by comparing the difference in mortality rates of birds that have not been oiled (affected) to birds that have been oiled, cleaned and subsequently released. The study showed that there was no significant difference in the death rate of the two groups, and that up to 87% of

rehabilitated penguins returned to their breeding colonies. Another study (Wolfaardt & Nel 2003) showed that the breeding productivity of the rehabilitated birds was on average no different than that of other penguins not affected by an oil spill.

2.6. Avian Malaria at SANCCOB

Although the rehabilitation of penguins has been very successful, mortalities during the summer as a result of avian malaria infection have reduced the rehabilitation efforts considerably. Avian malaria is a known cause of mortalities in captive penguins kept in open-air facilities (Cranfield *et al*., 1990). During rehabilitation at SANCCOB, mortalities due to avian malaria can range from 50-70% during the summer months and approximately 27% of deaths of admitted penguins are attributed to malaria each year (Parsons 2001).

During 2001 and 2002, 34% and 17% respectively of penguins admitted to SANCCOB were diagnosed positive for malaria at some stage during their stay at the rehabilitation centre. Over these two years 23% (109 out of 467) of penguin deaths were attributed to malaria, with mortality associated with malaria in the winter months considerably less than in the summer months (Parsons & Underhill 2004). Diagnosis of malaria as a cause of death is generally confirmed with a positive blood smear, a post-mortem evaluation, a positive kidney impression smear, or histopathology. 79% of penguins were diagnosed positive for malaria at some stage in 2001 and subsequently released, while in 2002 this percentage was 74%. This number is comparable with the overall release rate of 74% and therefore malaria in penguins does not affect the overall release rate. However, penguins that were diagnosed positive at some period during their stay at the centre had on average stays about 70% longer than those found to be negative. The reasons for these longer stays were both because they were ill and because the 10-day treatment extended their stay (Parsons & Underhill 2004).

In conclusion, it can be said that malaria has a considerable effect on the effectiveness of rehabilitation and places a considerable economic burden on the facility by extending the rehabilitation period of penguins. In view of the fact that malaria plays such a central role in penguin rehabilitation and was the primary motivation for this study, an overview of malaria will be given in the next chapter.

CHAPTER 3

MALARIA

3.1. Introduction

Malaria is an infectious disease caused by parasites of the genus *Plasmodium* and remains one of the major health problems in tropical and subtropical regions. It is a parasitic infection transmitted by mosquitoes, infecting reptiles, birds and mammals. Several species of *Plasmodium* have received considerable attention for their medical (e.g. *P. falciparum* and *P. vivax* in man) or veterinary (e.g. *P. gallinaceum* in chickens), or ecological (e.g. *P. relictum* in birds) importance. The genus is estimated to include at least 172 species, of which 89 occur in reptiles, 32 in birds and 51 in mammals, although the most research emphasis falls upon *P. falciparum*, the agent of lethal malaria in man (Paul *et al*., 2003).

Malaria is one of the most prevalent human infections worldwide. Attempts to eradicate malaria have been unsuccessful and efforts to control the disease are becoming less successful because of anti-malarial drug resistance in the parasite and insecticide resistance in mosquitoes. The major problem, however, is the extraordinary biology of this organism. The malaria parasite is an extremely small, haploid but genomically complicated eukaryote, able to change its gene expression to produce a sequence of structurally different forms, capable of surviving in different environments: liver and red blood cells in humans; gut, vascular system and salivary glands in the mosquito. In humans, the parasite lives mainly within cells, protected there from most circulating antibodies, and evades the host's immune attack on accessible parasite antigens by varying the expression of their genes. The parasite also causes infected red blood cells to adhere to blood vessel walls to minimize destruction of these cells by the liver and spleen. Another cause of this parasite's success is its ability to distribute itself *via* a highly prolific insect vector, the mosquito, with a high breeding rate and a high rate of evolution, for example, of insecticide resistance (Garnham 1966, Frost *et al*., 1976, Bannister & Mitchell 2003, Paul *et al*., 2003, Suh *et al*., 2004).

3.2. Malaria parasite life cycle

3.2.1. Exo-erythrocytic liver stages

When an infected female insect vector takes a blood meal, she injects saliva into the vertebrate host (Figure 3.1). The saliva contains an anaesthetic, an anti-coagulant and, if infected with *Plasmodium*, the parasite sporozoite stages that invade host cells. The sporozoite must evade the vertebrate immune system and invade host cells. The initial target cell varies. The liver cells are the targets in mammals, whereas in birds and reptiles this pre-erythrocytic cycle is more complex, involving several rounds of invasion and asexual multiplication, initially in skin macrophages before spreading throughout the body (Meis & Verhave 1988). The sporozoites undergo asexual proliferation in these host cells producing tens of thousands of merozoite stage parasites within a week. At maturation, these merozoites are released into the blood system where they invade erythrocytes, initiating the erythrocytic cycle (Garnham 1966, Kappe *et al*., 2003, Paul *et al*., 2003).

3.2.2. Asexual erythrocytic stages

The free merozoite is very small (~1.2 μm long), but it contains everything necessary to invade and establish itself in the red blood cells (RBC). To succeed in getting into an uninfected RBC, the merozoite has to rapidly select and adhere to it, then enter and seal itself inside. The merozoite now changes to the ring stage. Having invaded a RBC, the parasite spreads itself into a thin biconcave disc, giving it the appearance of a ring in Giemsa-stained blood smears. The parasite is sealed off in a membrane-lined cavity, the parasitophorous vacuole, within the RBC and feeds on haemoglobin through its cytostome, as well as taking up nutrients transported in from the plasma. The haem products of haemoglobin digestion crystallize into particles of dark pigment, haemozoin, scattered within the food vacuole. The parasite starts to synthesize molecules specific to its stage, some of which are exported into the RBC, modifying the RBC membrane, enabling it to adhere to the endothelium of blood vessels. The ring grows into the more rounded trophozoite stage (Fig. 3.2A), which is the period of most active feeding, growth and RBC modification. New molecules are synthesized and exported into the RBC, changing its structure and increasing its permeability to nutrients. The parasite now forms a schizont where the nucleus now divides to form ~ 16 nuclei (Fig. (3.2B).

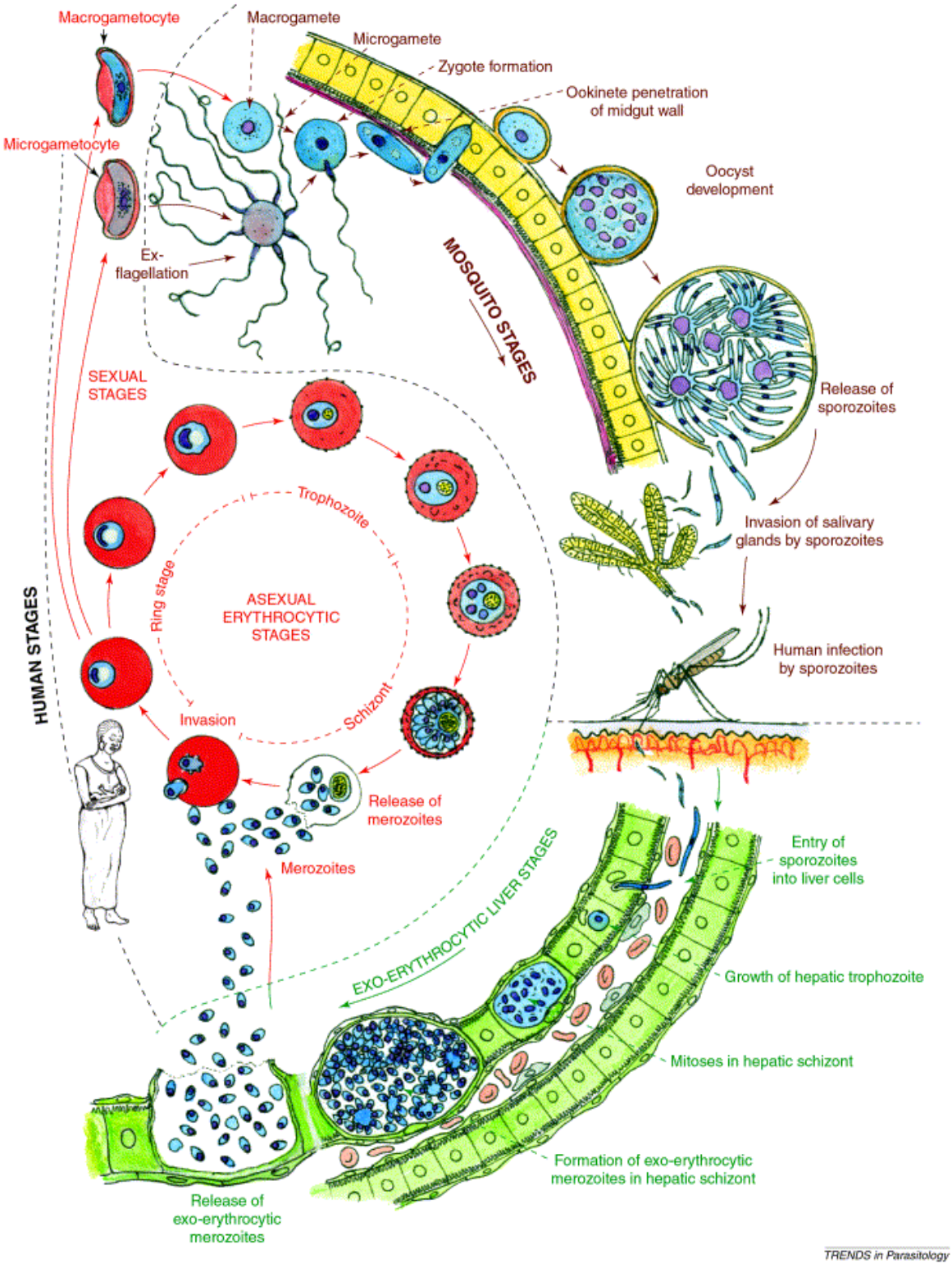


Figure 3.1: The life cycle of malaria parasite (Bannister & Mitchell 2003).

Merozoites appear around the periphery of the schizont, each receiving a nucleus. The merozoites eventually pinch off from the residual body of cytoplasm and are released into the bloodstream when the RBC membrane and the parasitophorous vacuolar membrane lyse. The free merozoite invades further erythrocytes (RBCs). This cycle

generally occurs every 24 -72 h, according to the *Plasmodium* species, and these asexual blood stages are responsible for disease. Re-infection of exo-erythrocytic cells from blood-stages occurs in avian and saurian *Plasmodium* species (Garnham 1966, Bannister & Mitchell 2003).

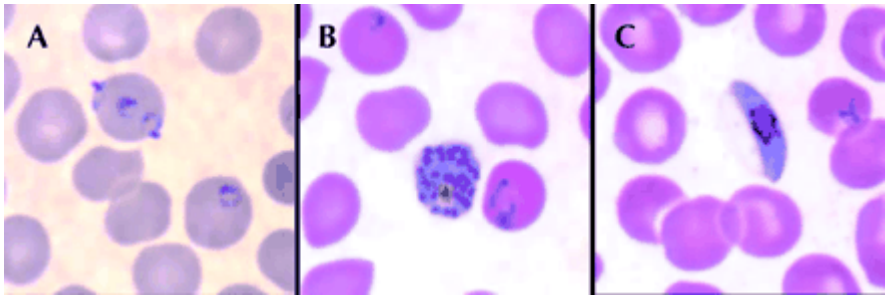


Figure 3.2: Stages in the life cycle of *Plasmodium falciparum* (Suh 2004). A: Trophozoite stage. B: Mature schizont. C: Gametocyte.

3.2.3. Sexual stages

Transmission of *Plasmodium* from vertebrate host to the insect vector is mediated solely by the sexual stages, the gametocytes, which are distinguishable as males and females (Fig. 3.2C). At some point during the course of infection the merozoite stages grow, but do not divide and produce gametocytes, which are gamete precursors. Mature gametocytes are arrested in G_0 of the cell cycle in the vertebrate host blood until another female ingest them, whereupon they transform into gametes. Gametogenesis occurs within 10-15 minutes after uptake in the blood meal. This happens as a response to the drops in temperature and pH associated with the different host factors. The process of digestion destroys any asexual parasites that happen to be present, while gametocytes quickly shed their erythrocyte envelopes and undergo maturation into the respective male and female gametes. Male gametocytes undergo exflaggellation producing up to eight male gametes, while each female gamete only produces one female gamete. The male gamete must actively swim to find and fertilize the female gamete forming a zygote (Garnham 1966, Sinden 2002, Bannister & Mitchell 2003, Paul *et al*., 2003).

3.2.4. Mosquito stages

The zygote transforms into a mobile ookinete that forces its way through the brush border of an epithelial cell of the mid-gut and enters the cell by liquefy its wall. Here it secretes a thin cyst wall and proceeds to mature. After 8 – 15 days, depending on the *Plasmodium* species, the mature oocyst releases several thousand sporozoites, which invade the salivary glands of the mosquito and are injected into the vertebrate host during her blood meal, initiating another life cycle (Garnham 1966, Sinden 2002, Kappe *et al*., 2003, Paul *et al*., 2003).

3.3. Avian Malaria in the African Penguin

3.3.1. Avian Malaria

Malaria in birds, like in the human variant, requires mosquitoes as a vector. Avian *Plasmodium* is found in every continent except the Antarctic, and probably every country of the world. Such a diverse distribution can easily be explained by the vast migratory flights of birds. Parasites are carried across oceans and deserts, and birds are exposed to mosquitoes of all varieties. The infected foci are left behind at different places along the migration routes, from which the parasites are spread amongst the non-migratory birds by local vectors (Garnham 1966). Avian malaria commonly infects wild birds, but can also infect domestic fowl and 'cage birds' when suitable vectors and wild reservoir hosts are present. The identification and classification of malarial parasites is complex. Twelve subgenera of plasmodia are recognized: 3 in mammals, 4 in birds and 5 in reptiles. Avian subgenera include *Haemamoeba, Giovannoliaia*, *Novella* and *Huffia* (Redig *et al*., 1993, Atkinson 2001). The mammalian malaria *P. falciparum* is significantly more related to avian parasites than to other parasites infecting mammals and it has been hypothesised that *P. falciparum* is derived from avian *Plasmodium* species (McCutchan *et al*., 1984, Brooks & McLennan 1992). A complex of more than 30 species of *Plasmodium*, which differ widely in host range, geographical distribution, vectors and pathogenicity, are responsible for avian malaria infections (Garnham 1979). The parasite is not very pathogenic in birds that have evolved with malaria, often causing no clinical signs. However, in species of birds that have not evolved with malaria, it causes varying degrees of pathology and mortalities.

These bird species are usually in dry, cold or windy areas where the vector does not occur (Cranfield *et al*., 1990).

3.3.2. Malaria in the penguin

The first avian malaria case in a penguin was discovered in an African Penguin from Saldanha Bay (South Africa) in 1927 (Fantham & Porter 1944). In 1992, Brossy reported a 0.7% prevalence of avian malaria in wild African Penguins from Saldanha Bay. However, oiled and injured wild penguins rescued along the southern SA coast and rehabilitated at SANCCOB had a prevalence of 22%. Avian malaria is the main cause of mortality in outdoor penguin exhibits, causing 50% or greater mortality in untreated juvenile and adult penguins when first exposed to the vector (Griner 1974, Cranfield *et al*., 1990). According to Fantham and Porter (1944) the parasitemia prevalence of wild African Penguins was considerably lower than expected considering the abundance of *Culex* mosquito vectors and the social behaviour of the penguins. They explained this phenomenon by low gametocytemia, penguin age-related immunity to malaria, mosquito-impeding feathers and escape into water of penguins from mosquitoes. However, when birds are kept in restricted areas, endemic malaria may be transmitted to them because penguins in captivity do not spend the night in water, but remain huddled in pens where mosquitoes can easily bite through the bare skin around their eyes and the webs of the feet (Brossy 1992). It is doubtful that malaria in penguins would occur in the absence of infection in wild birds, since penguin infection occurs during periods of seasonally high infection rates in wild birds. Nonetheless, penguins cannot be ruled out as potential reservoirs, since primary penguin infections result in parasitemias persisting for up to 3 weeks. Recovered penguins rarely exhibit circulating parasites and are unlikely to serve as carriers of gametocytes for mosquito infections (Beier & Stoskopf 1980).

3.3.3. Parasites

Three types of malaria, *Plasmodium relictum, P. elongatum* and *P. juxtanucleare* can infect African Penguins. While *P. relictum* and *P. elongatum* occur naturally in breeding colonies (Bennett *et al*., 1993), *P. juxtanucleare* has recently been found at SANCCOB (Grim *et al*., 2003). *P. relictum* and *P. elongatum* are two of the most common avian malarial parasites (Garnham 1966) and a feature of these two parasites in African

Penguins is that the virulence of *P. relictum* has always been higher than that of *P. elongatum* (Graczyk *et al*., 1994c).

3.3.3.1. *Plasmodium relictum*

P. relictum (subgenus *Haemamoeba*) has one of the widest host ranges of avian plasmodia, occurring naturally in 70 different avian families and 359 species of wild birds. *Culex* species such as *C. quinquefasciatus*, *C. tarsalis* and *C. stigmatasoma* are proved natural vectors in Hawaii and California, although few epidemiological studies of *P. relictum* have been performed and natural vectors in other parts of its range are unknown (Atkinson 2001). *P. relictum* is a virulent parasite which causes an unknown amount of damage to the bird life of the world (Garnham 1966).

The wide distribution of *P. relictum* in different birds and mosquitoes led to speciation, during which process many strains of parasites became established with fixed characters of their own, while remaining antigenically similar. One of these subspecies, *P. relictum sphenisdidae*, has been reported by Fantham and Porter (1944) in African Penguins from Saldanha Bay. This parasite has also been found in other penguin species such as yellow-crowned penguins *Eudyptes antipodes*, rock-hopper penguins *E. cristatus* and the king penguin *Aptenodytes patagonica* (Garnham 1966).

3.3.3.2. *Plasmodium elongatum*

The subgenus *Huffia* has only two species and differs from all other avian malaria parasites in that its schizogony may occur in primitive blood-forming cells (Laird 1998). *P. elongatum* (subgenus *Huffia*) also has a wide host range, occurring in 21 different families and 59 species of birds and occurs in North and South America, Europe and Africa (Atkinson 2001). It is well established that numerous species of wild birds harbour plasmodial infections including *P. elongatum*. Seven different mosquito vectors are recognized as being capable of transmitting this organism, including *Culex* spp. mosquitoes that have been reported from the coastal areas that serve as natural habitat of African Penguins (Fleischman *et al*., 1968).

3.3.3.3. *Plasmodium juxtanucleare*

P. juxtanucleare (subgenus *Novyella*) occurs in Mexico, Brazil, Uruguay, Sri Lanka, Philipines, Taiwan, Japan, Malaysia, South Africa and Tanzania. The natural hosts for

P. juxtanucleare are jungle fowl (*Gallus lafayetti*), domestic hens (*Gallus gallus domesticus*), and turkeys (*Meleagris gallopavo*) and all reported natural infections leading to disease have been described in gallinaceous species (Atkinson 2001). Grim *et al.* (2003) identified *P. juxtanucleare* in African Penguins that were determined to have died from malaria at SANCCOB and is the first identification of this species associated with mortality in African Penguins and also as a pathogen in non-gallinaceous species (Grim *et al*., 2003). Proved natural vectors include *Culex* species such as *C. sitiens, C. annulus, and C. gelidus*.

3.3.4. Life cycle of the avian malaria parasite

The life cycle of avian malaria parasites differs to some extent from the human malaria parasite life cycle. In birds the life cycle, as in mammals, commences when infective sporozoites are inoculated into a susceptible host by a mosquito vector. In mammals the sporozoites invade liver cells, while in birds, the first stage of exoerythrocytic schizogony occurs in the tissue macrophages adjacent to the site of the mosquito bite. The secondary schizonts produce and release merozoites that infect reticuloendothelial tissues in organs throughout the body, creating secondary sites of exoerythrocytic schizogony or merogony. Avian plasmodia typically undergo three generations of exoerythrocytic or tissue reproduction, producing cryptozoites, metacryptozoites and phanerozoites in each successive stage over a period of about 72 hours. Phanerozoites leave the tissues and penetrate membranes of the RBCs, beginning the stages of erythrocytic activity. There are three possible outcomes of erythrocytic merogony. The trophozoites, as in mammals, can develop into either the sexual stage of gametocytes or the asexual stage of schizonts (Atkinson 2001, Cranfield 1990). The third possibility in contrast to mammalian plasmodia is the formation of merozoites that can re-infect tissues and re-initiate the tissue phase. It has also been shown that gametogenesis can occur directly from exoerythrocytic forms (Redig 1993).

3.3.5. Clinical signs

Avian malaria infection therefore has a tissue phase as well as a blood phase. The tissue phase of the infection causes tissue damage and is therefore responsible for the clinical signs of the disease, whereas the blood stage does not cause enough destruction to cause clinical anaemia in penguins. This is not the case in gyrfalcons

where more than 50% of the red cells will contain parasites and packed cell volumes can be reduced to 20% (Redig *et al*., 1993). *P. relictum* and *P. elongatum* are both capable of rapidly causing fatal disease in penguins. Premonitory signs are often subtle and frequently lacking altogether (Stoskopf & Beier 1979). The clinical signs include paleness, anoxia, depression, vomiting, breathing difficulty, regurgitation and death and gross pathology reveals an enlarged spleen, swollen liver, and edematous lungs (Cranfield *et al*., 1990).

Exoerythrocytic forms of *P. elongatum* cause extensive pathological changes in African Penguins and Stoskopf and Beier (1979) confirmed that peripheral parasitemias tend to be low in these birds, usually less than 0.01%. They also demonstrated that the interval between the onset of clinical signs and death could be as short as a few hours. Clinical signs are often non-specific and can be confused with other penguin diseases such as aspergillosis and bacterial gastroenteritis. Some birds seem to remain healthy despite of infection, suggesting that some *Plasmodium* species are more virulent than others.

3.3.6. Pathogenicity

Significant pathological effects of avian malaria are associated with both tissue and erythrocytic stages of infections, although in African Penguins the erythrocytic stage does not have such an immense effect as the tissue phase.

After sporozoites gain access to a suitable host, species of avian malaria develop in tissues of mesodermal origin, including endothelial cells lining capillaries, cells of the haemopoietic system, and cells of the lymphoid-macrophage system. Developing schizonts may restrict or completely block blood flow to vital organs, such as the brain and spleen, during heavy infections. Schizonts can also develop in cells of the heamopoietic system and cause severe anaemia, as is the case with *P. elongatum*. Serious disease and death can therefore occur prior to the appearance of parasites in the peripheral blood cells. Since the parasites have not yet invaded circulating blood, some characteristic gross pathological signs may not be evident. These include enlargement and discolouration of the liver and spleen. Significant pathological effects have been attributed to pre-erythrocytic schizonts and many of these effects may be secondary to blockage of blood flow and related to tissue necrosis.

RBC destruction and anaemia associated with erythrocytic schizogony are the most severe pathological consequences of infection with most species of *Plasmodium*. Infected RBC rupture during the release of merozoites from intracellular schizonts and can be removed by cells of the reticulo-endothelial system, particularly macrophages from the spleen, liver and bone marrow. Severe anaemia may result when destruction and removal of infected erythrocytes is not balanced by the synthesis and release of immature erythroblasts. Changes in plasma chemistry because of RBC destruction can result in decreases in plasma pH and increases in plasma proteins such as gammaglobulins and fibrin. These changes can reduce oxygen-binding capacity of haemoglobin and slow blood flow in capillary beds of major organs (Atkinson and van Riper, 1991).

3.3.7. Diagnosis

Clinical signs, overall clinical impression and evaluation of a blood smear are the main elements employed for establishing diagnosis. Blood smear evaluation is definitive, providing the organisms are present in peripheral blood. Clinical experience has shown that erythrocytic forms are present only after the disease is well established through the tissue phase and can be missed easily since the period of time that the erythrocytic forms can be found in the peripheral blood, is very short. Both asexual and sexual stages of the parasite can be seen on a thin blood smear. The asexual stages involve the young trophozoites, mature trophozoites and schizonts of which *P. elongatum* and *P. relictum* are impossible to differentiate. The species can, however, be identified by the sexual gametocyte (Cranfield *et al*., 1990).

In *P. relictum*, the gametocyte is round and takes up a large portion of the cell pushing the nucleus out of the way and often at 90 degrees to the normal position, while the gametocyte of *P. elongatum* is an elongated worm-like form that is wrapped around the nucleus of the cell (Cranfield *et al*., 1990). The appearance of *P. juxtanucleare* gametocytes can easily be misidentified as *P. relictum* because there is occasional displacement of the nucleus. Fortunately, other blood stages of *P. juxtanucleare* can be used for identification. The most distinctive blood-stage is the merozoite, found directly adjacent to the RBC nucleus, with little cytoplasm present (Grim *et al*., 2003).

Other methods for avian malaria diagnosis include isodiagnosis and haematological parameters as indicators. Isodiagnosis by inoculation of infected blood into susceptible hosts such as Pekin and Muscovy ducks has been used in avian malaria research to increase the sensitivity of blood smear diagnosis (Herman *et al*., 1966, Manwell & Hatheway 1943). However, the false negative results and the prohibitively long time required to read the tests make it undesirable as a clinical tool (Cranfield *et al*., 1990).

Stoskopf and Beier (1979) suggested haematological parameters as indicators for avian malaria infection. They noted that when a penguin had malaria, it often had a greater than 20 $x10^3/\mu L$ total white blood cell (WBC) count, whereas the relative lymphocytosis (LYMPHS) was greater than 60%. Graczyk *et al*. (1994c) undertook a study to evaluate the applicability of WBC counts and LYMPHS for diagnosis of avian malaria in African Penguins. They found that even though WBC and LYMPHS are valid indicators of avian malaria, individual variations were so high that diagnosis of infection using these parameters would not be accurate.

3.3.8. Treatment

The main objectives in the treatment of avian malaria are to eliminate the erythrocytic and tissue forms, and to provide protection from massive rupture of infected red blood cells as well as handling related stress during treatment. In some cases it is also necessary to provide the bird with a supply of functional red blood cells through transfusion, which can improve oxygen carrying capacity and help reduce the number of infected erythrocytes. Even though the choice of pharmalogical agents is narrow, a great deal is known about the efficacy, pharmacokinetics and dosing, for the reason that avian malaria-causing organisms have served as models for testing of human anti-malarial drugs, for cure as well as prevention.

Two agents have emerged for treating avian malaria, chloroquine and primaquine (primaquine phosphate). The former is effective against erythrocytic forms and the latter against the tissue forms (Redig *et al*., 1993). The effectiveness of this drug combination for treating avian malaria in penguins has been reported by Stoskopf and Beier (1979). However, despite the reasonable success in treating clinical cases it is clear that the parasite is not cleared from the system and most recovered birds may have lifelong infections (Cranfield *et al*., 1990).

3.3.9. Immunity

In naturally transmitted malaria infections, although initial parasitemias can be acute and potentially life threatening, host immunity controls parasitemia and extended periods of chronic infection follow before parasites are completely eliminated (Druilhe & Perignon, 1997). Control of initial parasiteamia by the host means that the parasites survive long enough for gametogenesis and transmission to occur. Chronic infection ensures that the parasites persist in the host for the extended periods, which can link possible rainy seasons, mosquito development and thus parasite transmission. How the malaria parasite survives in the face of host immunity remains one of the fundamental issues in malaria research. Hosts can show a partial or a complete resistance to plasmodial infection, either because they possess innate immunity or because they possess acquired immunity (Brown, 1969).

3.3.9.1. Innate immunity

Hosts with no previous experience of malarial infection display innate immunity. This includes the effect of natural antibodies, normal scavenging function of phagocytes or unsuitability of the host for the growth of the *Plasmodium*. Vertebrates show varying degrees of innate immunity. Some show complete resistance, while others can show a phasic resistance where, for example, normal development of exoerythrocytic stages occurs but no erythrocytes are infected. Another possibility is incomplete resistance where development of all stages of infection occurs, but parasite multiplication is restricted. The mechanisms of innate immunity are not well defined; nevertheless, factors that can affect observed infection, include the host species, its genetic constitution, age and environment. Immunity may also be non-specifically acquired by infection of the host with another organism (Brown, 1969).

3.3.9.2. Immunity acquired through infection

Acquired immunity is a state of partial or complete resistance existing in a previously susceptible host. Such immunity may be non-specific or specific. An example of non-specific immunity occurs when infection of the host with another organism may induce hyper-reactivity of the reticuloendothelial system. Specific acquired immunity derives from the recognition of and the response to plasmodial antigen. It may be naturally acquired by the passive transfer of antibody from mother to fetus or as a result of

infection, or artificially induced by immunization. Antiplasmodial immunity is restricted in its specificity and may be effective only against a strain of a given species of *Plasmodium*. Apart from the strain and species specificity of acquired antiplasmodial immunity, there is evidence that those stages of the life cycle that are immunogenic stimulate an immune response specific for themselves (Brown, 1969).

The circumsporozoite protein is the most abundant protein on the sporozoite. Together with the trombospondin related adhesive protein it participates in binding to the target cells and is the target of neutralising antibodies. The immunodominant B-cell is highly conserved within each plasmodial species. Sporozoites that have not been blocked by antibodies will infect host liver or other target cells, where they differentiate and replicate before lysing the cells. During this time cytotoxic cells and cells capable of secreting IFN-γ can promote the elimination of intracellular parasites.

During the erythrocytic cycle, protection is partly antibody mediated, but IFN-γ production and T cell proliferation in response to blood-stage antigens are also associated with protection. Antibodies inhibit parasite growth by causing complement-mediated lysis of infected red cells and blocking red cell invasion. T-cell secretion of IFN-γ help induce cytophilic Immunoglobulin G (IgG) blood-stage-specific antibodies and assist in antibody-dependant cellular inhibitory mechanisms.

Pre-erythrocytic and erythrocytic parasites are both capable of interfering with the induction of T-cell responses. Parasitised red cells can interact with antigen-presenting cells, such as macrophages and dendritic cells, thereby inhibiting their activation. Furthermore, memory T cells specific for malaria blood-stage antigens, exhibit a pattern of accelerated apoptotic cell death during blood-stage infection (Plebanski & Hill, 2000, Garraud *et al*., 2003, Pouniotis *et al*., 2004).

3.3.9.3. Maternal Antibodies

Maternal or parental antibody transfers are potential mechanisms of equipping chicks with parent-derived immunoglobulins. Cases of maternal-fetal antibody transfer have been described in chickens, mallard ducklings, cockatoos and parrots. Maternal antibodies are usually sequestered from the maternal circulation by the developing oocyte and subsequently transported from the egg yolk across the yolk sac membrane into the embryonic circulation. Transferred antibodies are predominantly IgG, while

transfer of IgA and IgM usually occurs at substantially lower levels (Buxton 1952, Kramer *et al.*, 1970, Rose and Cho, 1974, Kowalczyk *et al.*, 1985).

Immunoglobulins are also transmitted prenatally in African Penguins. Graczyk *et al*. (1994a) and Graczyk and Cranfield (1995) described egg-yolk transfer of *Plasmodium* spp. IgG and *Aspergillus* spp. IgG in captive African Penguins. Graczyk *et al.* (1994a) found that all penguin neonates were positive for anti-*Plasmodium* immunoglobulins while housed in a mosquito-free environment. The chicks had a high anti-*Plasmodium* antibody titre after hatching, though after 2 months, the level of maternal antibodies was close to zero.

3.3.9.4. Recrudescence

Recrudescence by definition occurs in the absence of reinfection and shows as a reappearance of patent blood infection after primary parasitemic attack has subsided. Immunity does not completely eliminate the tissue forms and herein lies the basis of continued immunity, as well as seasonal and stress related recrudescence of the organism. Avian malaria cannot be cleared from the body and therefore once penguins become infected, they remain infected for life (Manwell 1934, Cranfield *et al*.,1990). If a penguin survives the first infection with avian malaria its immune system appears to be capable of reducing the number of parasites to sub-clinical levels.

Stress factors such as nutritional-, environmental-, or migration stress can induce recrudescence of parasites in penguins. Recrudescence and relapses of malarial parasites in various species of wild birds have been reported (Cranfield *et al*., 1994). A few hypotheses have been proposed to explain this phenomenon (Brown, 1969). One of these suggests that exoerythrocytic (tissue) stages continuously release merozoites into the circulating blood, and these allow the parasite population to recover when premunition to them declines. Another hypothesis proposes that dormant sporozoites or pre-erythrocytic forms survive in endothelial tissues and can later cause parasite recrudescence under a specific stimulus.

3.3.10. Prevention

Prevention of malaria is based on two approaches namely vector exclusion and prophylactic treatment. Where susceptible species are housed at a time of the year and

a place where vectors and reservoirs are present, they must be protected from insects during the time of day when these vectors are active. Prophylactic treatment consists of a once weekly single treatment with either primaquine or chloroquine/primaquine combination. Treatment should commence one month before and continue until one month after the insect season and periodic blood samples should be taken for monitoring purposes.

3.4. Potential impact on conservation

Offering aid to the sick and injured is a normal humanitarian reaction which is widely applied to animals as well as humans and any animal or bird unable to fend for itself will readily find helping hands. An increasingly large group of people take animals or birds out of the wild, either to allow healing of disease or to rear fledglings or cubs, which have lost their parents. Even though these efforts are not always successful, sometimes the creature can be released and it will return to its natural habitat. Unfortunately if they succeed, they produce a fresh set of problems of which the most important is the spread of diseases acquired in captivity into the wild population.

The destructive effect of avian malaria as an introduced disease is evident in the history of Hawaiian land birds. A larger portion of the endemic bird species of the Hawaiian Islands have become extinct in historical times than in any other comparable region of the world. Malaria has had and is presently having a significant negative impact upon the native Hawaiian avifauna. Mosquitoes were absent from this region until 1826 when a sailing vessel stopped at Lahaina, Mauaia, and in the course of refilling the water kegs, introduced *Culex pipiens*, which subsequently invaded the others islands of the group. About the same time, many of the indigenous birds vanished, and Warner (1961) suggested that the mosquito transmitted a lethal form of avian malaria from infected migrant birds to the local susceptible ones, which were largely wiped out. However, avian malaria was not noted until 1939 and Van Riper *et al.* (1986) proposed that even though the mosquito vector was present, avian malaria did not reach epizootic proportions in Hawaii until the 1900's, following the numerous releases of introduced birds, particularly from Asia. By 1920, a large enough pool of infected introduced avian hosts was present in Hawaii to begin the spread of malaria to native birds species. The reduction of the Hawaiian avifauna prior to 1910 is therefore not thought to be because

of avian malaria, but rather habitat destruction by humans and introduced ungulates, indiscriminate killing of birds and introduced predators. If disease did play a role in the initial decline of birds, a logical explanation would be a virus, such as Avian Pox (Van Riper *et al.*, 1986).

Avian malaria is found in a number of common mainland flying birds in the Western Cape. The vector, a culicine mosquito, is common in the Western Cape; so cross-infection from flying bird to penguin is apparently easy and common. As mentioned in previous sections, African Penguins can be infected with three types of malaria: *Plasmodium relictum, P. elongatum* and *P. juxtanucleare*. While, *P. relictum* and *P. elongatum* have been found to occur naturally in the breeding colonies, *P. juxtanucleare* has been found at SANCCOB, and is the first identification of this species associated with mortality in African Penguins (Grim *et al*., 2003). *P. relictum* and *P. elongatum* is endemic in wild penguins and this can confer a low degree of cross-immunity to the mainland malaria, although the morbidity and mortality suffered by penguins with 'mainland' malaria shows that any cross-immunity is very limited. Penguins that are released from SANCCOB tend to return to their colonies and if these released birds carry non-endemic malarial parasites they present a potential hazard to the rest of the colony. Diseases that penguins and other birds can spread to their natural environments after release include Newcastle disease, aspergillosis, leucocytozoonosis and perhaps others we do not yet know about (Brossy *et al.*, 1999). Of far greater concern is avian malaria. This is especially true when considering how vulnerable penguins are to this disease, as well as the disastrous consequences that introduced malaria can have for immunologically naïve, endemic birds in island systems such as Hawaii.

In conclusion, avian malaria can have a devastating effect on immunologically naïve birds. In view of the fact that mainland avian malaria can be introduced into African Penguin breeding colonies, an accurate assessment of the malaria occurring at SANCCOB and naturally occurring exposure to avian malaria in wild African Penguins is vital to understanding the consequence of releasing penguins infected with mainland avian malaria into the wild. For that reason, an investigation into avian malaria at SANCCOB and in the breeding colonies was attempted in this study, using immunological and molecular techniques. These techniques will be discussed in the next chapter.

CHAPTER 4

MOLECULAR TECHNOLOGY AND AVIAN MALARIA

4.1. Introduction

For more than a century, malaria researchers and clinicians have used stained blood smears to diagnose malaria and to identify the organism causing malaria. However, various problems are associated with microscopy as a diagnostic tool. For example, some parasites are morphologically similar or very small and difficult to stain and detect. Microscopy is also extremely labour intensive, especially when a large number of samples needs to be screened in a relatively short time, such as during epidemiological studies.

Avian malaria diagnosis is extremely important since it is often too late for treatment when the clinical signs appear (Cranfield *et al*., 1995). However, due to the persistence of tissue schizogony, parasites may completely abandon the RBC and move to endothelium or hemopoietic tissue, or remain in RBC at a level undetectable by the blood smear method (Garnham, 1966). These factors, along with extension of length of pre- and exoerythrocytic schizogony, make examination of blood smears inaccurate for diagnosis (Graczyk *et al*., 1993).

In order to overcome some of the difficulties encountered while using microscopy for parasite diagnosis, immunological and molecular techniques have been developed. The enzyme-linked immunosorbent assays (ELISA) allows the measurement of specific antibodies and therefore immune responses can be tested. Furthermore, the development of the polymerase chain reaction (PCR) has provided new ways of studying the malaria parasite, its vector and its host. For this reason, PCR and ELISA will be discussed fully in the next section.

4.2. Enzyme-linked immunosorbent assay

4.2.1. Introduction

The basis of all immunoassays is the interaction of antibodies and antigens. Yalow and Berson developed the most widely used immunoassay, the radioimmunoassay (RIA), in 1959. The principle of RIA is elegantly simple and has been used particularly in clinical laboratories to quantitate a wide variety of compounds. However, radioisotopes do have their drawbacks including, health risks, expensive equipment and strict regulatory control. These disadvantages encouraged the search for alternative non-isotopic immunoassays. In 1971, Engvall and Perlman described the use of enzyme-labelled reagents in the enzyme-linked immunosorbent assay. The ELISA is a well-known and widely used laboratory technique that is able to measure ligands present in small amounts in biological samples. The assay can measure both antigens and antibodies with a high degree of sensitivity and specificity. It has become the most popular immunoassay used in research laboratories. Some of its advantages include rapidity, inexpensiveness and safety.

Four forms of enzyme immunoassay have been developed. The antibody sandwich immunoassay, the antibody capture immunoassay and the antigen capture immunoassay are ELISAs for antigen detection, while the indirect antibody capture immunoassay is an ELISA for antibody detection (Goers, 1993).

The most common type of ELISA is the indirect antibody capture immunoassay (Figure 4.1. A). The basic procedure in setting up this ELISA is as follows; the antigen is coated onto a solid phase, samples containing the antibodies directed against the antigen are added, and the detection is carried out by an enzyme-labelled anti-antibody. The enzyme acts on an appropriate substrate, releasing a coloured compound that can be easily detected by a spectrophotometer.

The procedure can easily be adapted to cases where the analyte to be detected is not an antibody, but an antigen (antibody sandwich immunoassay, Figure 4.2. B). In this immunoassay, antigen-specific primary antibodies are adsorbed to the solid phase. Samples containing the antigen are added where after a second, labelled antibody must bind the antigen. Only multivalent antigens with repeating epitopes can be detected in

this assay, since binding of two antibodies to the antigen is required. Fortunately, this requirement is not normally a limitation for proteins, as they are almost always multivalent (Caponi & Migliorini 1983, Goers, 1993).

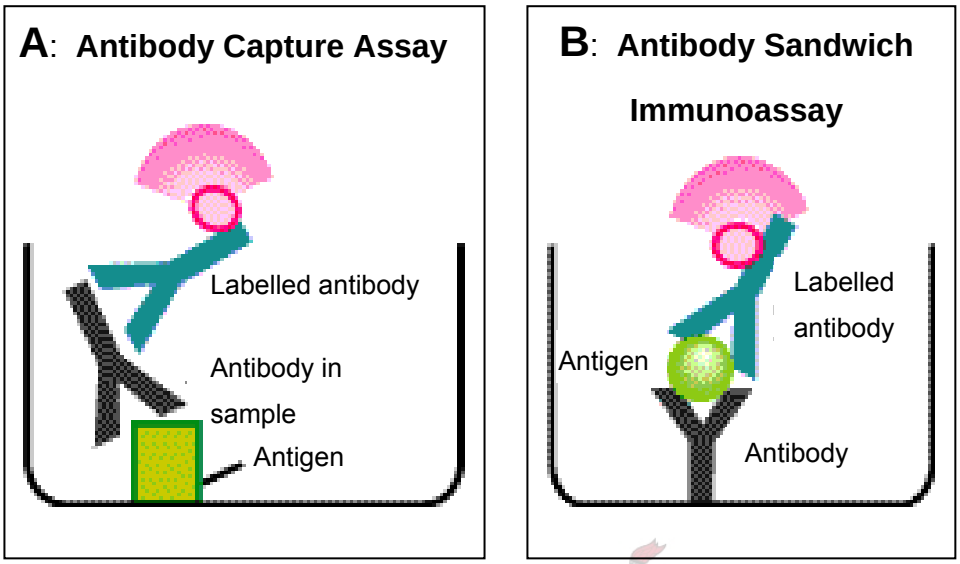


Figure 4.1: A. The antibody capture assay. The analyte to be detected is an antibody directed to the coated antigen. **B.** The antibody sandwich assay. The analyte to be detected is an antigen with multiple epitopes.

4.2.2. Solid-phase

Stable and reproducible binding of antigens or antibodies to a solid phase is essential for most resolution immunoassays. This allows easy manipulation of the antibody-antigen complex during the different steps. Commonly used solid phases include, nitrocellulose, polyvinylchloride, polystyrene, diazotised paper and activated beads (Table 4.1, Harlow and Lane, 1988).

The most convenient solid support for the ELISA reaction is the 96-well microtiter plate, which comes in a standardised form for use with most microtiter plate readers. Microtiter plates are usually made of polystyrene or polyvinylchloride. These plates are transparent and allow transmission of light with low background noise. Direct coating or passive adsorption often obtains a satisfactory result. The adsorption of molecules to a polystyrene surface is due to the intermolecular attraction forces such as van der Waals forces (Larson *et al*., 1987). Van der Waals mediated bonds are about 100 times weaker than ionic and covalent bonds. The relatively low binding capacity of this material can be enhanced by physical or chemical treatment, although this often

increases the background. Covalent bonding to proteins is accomplished by modifying plates chemically (Caponi & Migliorini 1983).

Table 4.1: Solid supports for immunoassays.

Support	Forms available	Method of binding
Nitrocellulose	Membranes Microtiter wells	Noncovalent
Polyvinylchloride	Microtiter plates, Sheets	Noncovalent
Polystyrene	Beads, Microtiter plates	Noncovalent
Diazotized paper	Sheets	Covalent, through free amino groups
Activated beads, numerous attachments possible	Beads	Covalent, through free amino groups

4.2.3. Saturation

After the adsorption of the antigen onto the plate, a saturation agent is added to the well in order to saturate areas of the plate not covered by the antigen. This will in principle prevent non-specific binding of the antibody in the sample plates. Casein and gelatin preparations contain proteins of different sizes and are therefore good saturating agents because they can efficiently cover those areas left uncovered by antigen. Bovine serum albumin (BSA) and newborn calf serum, containing relatively larger molecular weight components, are also used as saturating agents. Random lose packing of these larger proteins potentially can leave bare patches of unblocked plastic surface to which second stage reagents can bind, leading to a higher background value (Caponi & Migliorini 1983). However, at the concentration routinely used, i.e. 1% m/v, all of the above-mentioned proteins have been used successfully as saturating agents in ELISA.

4.2.4. Antibody or antigen binding

After saturation, the next step in the antibody-capture ELISA is the binding of antibody to antigen and in the antibody sandwich assay is the binding of antigen to antibody on the solid phase. In the former, the antibody-containing sample is added to an antigen-coated well and if the sample contains antigen-specific antibody, the antibody will bind

to the antigen and is detected by a labelled species-specific secondary antibody. The captured test antibody is therefore detected with a second labelled antibody (Goers 1993). In the antibody sandwich assay, an antigen-containing sample is added to an antibody coated well and if the sample contains the required antigen, the antigen will bind to the antibodies. The antigen is then detected with a second, labelled, antigen-specific antibody.

4.2.5. Detection

Antibodies modified with a label are often used to detect antigens in a variety of immunochemical techniques. The labelled antibody binds to the antigen and then is detected by using the label as a signal. The most common labels are radioactive isotopes, fluorescent labels and enzymes, although colloidal gold and heavy metals are also used. The choice of label depends on the end use: enzyme and radioactive labels are very effective in blot and ELISA techniques, while fluorescents and metal labels are particularly useful in flow cytometry and immunocytochemistry.

When using radioactive labels, any radioactive isotope that is easy to detect can be used to label antigens or antibodies. Proteins can be labelled *in vitro* by iodination or *in vivo* by growing cells in the presence of radioactive precursors. Radioactivity can be located by using ß- or γ-counters or by normal X-ray film detection methods. Fluorochromes may be bound to either antibodies or antigens *in vitro*. Measurement of the bound fluorochrome will require an appropriate detection device (Harlow and Lane, 1988).

When using enzyme labels, the detection system consists of an antibody labelled with an enzyme and directed to the analyte of the sample, plus its substrate. The antibody labelled with an enzyme combines the specific recognition properties of the antibodies with the high sensitivity characteristics of enzyme based analytic techniques. The substrate, when enzymatically cleaved, releases a coloured compound that can be detected by a spectrophotometer. A single enzyme can act on several molecules of substrate and therefore the catalysed reaction is amplified and the sensitivity of the assay is enhanced. Enzymes that are mostly used are horseradish peroxidase, calf intestinal alkaline phosphatase and β-galactosidase from *Escherichia coli*.

The avidin-biotin system can further be used to enhance the sensitivity of an ELISA. Biotin is a small vitamin that can be covalently bound to antibody or enzymes without affecting the antigen-binding capacity of the antibody or the activity of the enzyme (Geusdon, 1988). Avidin is a basic glycoprotein obtained from egg white, with a high affinity for biotin. In some cases streptavidin is preferred to avidin. Streptavidin is a protein very similar to avidin but is produced by bacteria. Antibodies can be biotinylated in a simple procedure without loss of binding capacity. The streptavidin linked to the enzyme label is added and finally a chromogenic reaction is carried out. The affinity of the (strept)avidin-biotin system is much higher than that of the antigen-antibody complex and together with the fact that a single molecule of streptavidin can bind four molecules of biotin; these two factors can aid in raising the sensitivity of the assay without a significant increase in background values (Caponi & Migliorini 1983).

Enzyme reactions are quantifiable by various measuring techniques that are determined by the respective substrate and the features of the resulting product. These measuring techniques include luminescence technology, fluorimetry, potentiometry, amperometry- and colorimetry. However, the most frequently applied technique is measuring a coloured product of a reaction, which is formed from a colourless substrate due to the catalytic activity of the marker enzyme. Chromogenic substrates for marker enzymes have the advantage of simple evaluation, either visually or by spectrophotometers. Main requirements are chromogen product with high extinction coefficients, high turnover rates of marker enzymes, stability after termination of reaction, and no or limited hazardous properties. Chromogenic products of green, red and blue colours are particularly suitable for visual evaluations.

Particularly sensitive as chromogens for peroxidase measurement are o-phenylenediamine, Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), tetramethylbenzidine, aminosalicylic acid and o-dianisidine. Tetramethylbenzidine and o-phenylenediamine are both highly sensitive but carcinogenic, while aminosalicylic acid and o-dianisidine are less sensitive than ABTS. ABTS is sensitive, gives very low background values and is therefore an ideal chromogen. For alkaline phosphatase, p-nitrophenyl-phosphate is regularly used as a chromogen, while activity determination of β-galactosidase is mostly carried out using o-nitrophenyl-galactopyranoside (Porstmann & Porstmann 1988).

4.2.7. ELISA for the detection of antibody levels to avian malaria

Graczyk *et al*. developed an ELISA for the detection of antibodies against *P. relictum* and *P. elongatum* (Graczyk *al et*., 1993). This ELISA is based on the cross reactivity between anti-*P. relictum* and anti-*P. elongatum* antibodies with antigens of *Plasmodium falciparum* and makes use of a recombinant circumsporozoite (CS) protein of *P. falciparum* as a capture antigen. Graczyk *et al*. (1994b, 1995) evaluated this ELISA for the diagnosis of avian malaria infections in African Penguins and found it to be an effective tool for diagnosing exposure to avian malaria in penguins.

The ELISA used in this study was based on the ELISA described by Graczyk *et al*. (1994b) and was developed by Botes (2004) in order to assess the natural immunity of penguins to avian malaria on entry into the SANCCOB facility and during the rehabilitation process (Figure 4.2). In this ELISA a synthetic peptide (with the sequence $(NANP)_4$) corresponding to the repeat region of the CS protein of *P. falciparum* was coupled to BSA. This peptide-BSA conjugate was used as the coating antigen. Casein was used as the blocking agent, and the anti-*Plasmodium* antibodies were detected by biotinylated rabbit anti-penguin antibodies. The biotin was subsequently detected by streptavidin coupled to horseradish-peroxidase. The horseradish-peroxidase was used as the enzyme label with ABTS as the final substrate. The absorbance is measured at 405 nm. This ELISA is an adaptation of the antibody capture assay as seen in figure 4.1. A.

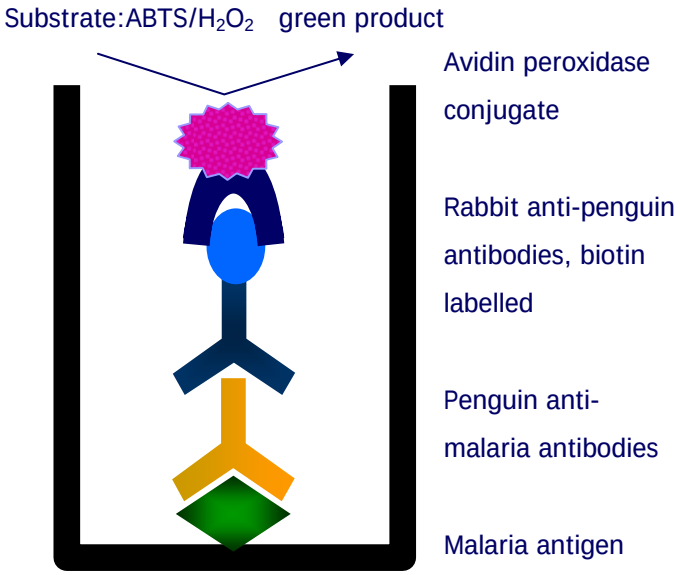


Figure 4.2: Enzyme linked immunosorbent assay (ELISA) for detection of antibodies against avian malaria in penguins.

4.3. Polymerase chain reaction

4.2.1. Introduction

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. The PCR method was invented and named by Mullis and colleagues at the Cetus Corporation (Mullis and Falcona, 1987), even though Khorana and colleages had described the principle in detail over a decade earlier (Kleppe *et al*., 1971, Panet and Khorana, 1974). The use of PCR was limited until heat-stable DNA polymerase became widely available (Chien *et al*., 1976). PCR has since rapidly become established as one of the most widely used techniques of molecular biology for the reason that it is a rapid, inexpensive, and simple means of producing micrograms of DNA from minute quantities of source material and is relatively tolerant of poor quality template (Taylor 1991).

During PCR, specific regions of DNA are amplified enzymatically through successive cycles (figure 4.4). Each cycle consists of three steps; in the first or denaturation step, double stranded DNA is denatured to produce two single stranded DNA strands. In the second or annealing step, two different oligonucleotides (primers) hybridise to complementary DNA sequences on each of the target single stranded DNA strands. In the third or extension step, the enzyme DNA polymerase catalyses the addition of deoxynucleotide triphosphates, which are present in the solution, to the two oligonucleotides in the 5' to 3' direction (Taylor 1991).

Each of the three steps is undertaken at different temperatures: usually the denaturation step at 94°C and extension occurs at 72°C. The annealing temperature depends on the length and the nucleotide content of the oligonucleotide used in the PCR. In subsequent cycles, both the original target DNA and newly synthesized fragments serve as templates. The heating and cooling cycles can be repeated and DNA will continue to accumulate exponentially until one of the reaction reagents is exhausted or the enzyme is unable to synthesize new DNA quickly enough. Amplification either stops or produces non-specific products after a certain number of cycles. The number of cycles required for optimum amplification varies depending on the amount of starting material and the efficiency of each amplification step (Taylor 1991).

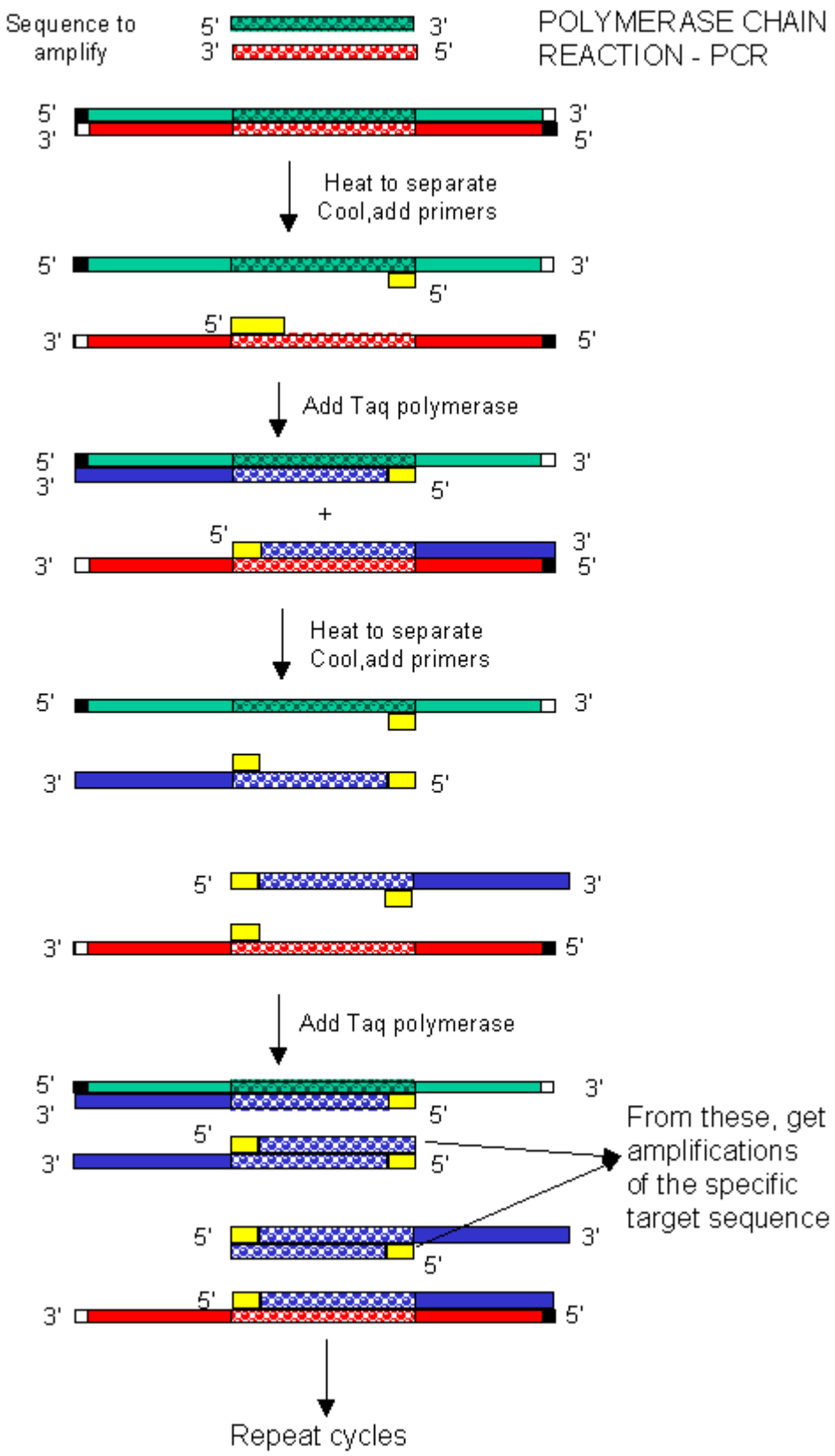


Figure 4.4: Polymerase chain reaction (PCR). Oligonucleotides complementary to a given DNA sequence prime the synthesis of only that sequence. Heat-stable Taq DNA polymerase survives many cycles of heating. Theoretically, the amount of the specific primed sequence is doubled in each cycle.

Each cycle of PCR doubles the amount of specific DNA resulting in a million-fold amplification of the target sequences after about 30 cycles. A final incubation step at the extension temperature results in fully double-stranded molecules from all emerging products.

The requirements of the reaction are straightforward: deoxynucleotides to provide both the energy and nucleosides for the synthesis of DNA, DNA polymerase, primer, template and buffer containing magnesium (Taylor 1991).

4.2.2. Reaction components

4.2.2.1. DNA polymerase

DNA polymerase uses single-stranded DNA as a template and makes a complementary strand by polymerising deoxynucleotides in the order specified by their base-pairing with bases in the template. Polymerization always proceeds from the 5' phosphate to the 3' terminal OH group of the growing DNA strand. DNA polymerases can only elongate pre-existing chains, since they cannot join two deoxyribonucleoside-5'-phosphates together to make the initial phosphodiester bond, and therefore a primer (oligonucleotide of approximately 20 nucleotides in length) is essential. Deoxynucleotide triphosphates can then attach to the free 3' terminal OH group of the primer as specified by the template sequence (Rolfs *et al*., 1992).

Three different DNA polymerases have been described in the eubacterium *Escherichia coli* with three different molecular structures and functions. These enzymes are numbered I, II and III, in order of their discovery. DNA polymerases I and II function principally in DNA repair, while DNA polymerase III is the chief DNA-replicating enzyme of *E. coli* (Garrett and Grisham, 1999).

The "historical" experiments on the PCR still used the thermolabile *E. coli* DNA Polymerase I, Klenow fragment (Mullis and Faloona, 1987). These experiments had the disadvantage that fresh, non-denatured aliquots of enzyme had to be added to the reaction mixture after each denaturation cycle. The identification of thermostable DNA polymerases made it possible to perform *in vitro* DNA amplification without having to add fresh enzyme after each cycle. The thermostable DNA polymerase from the eubacterium *Thermus aquaticus* (*Taq* polymerase) has been most widely studied. *T.*

aquaticus strain YT1, a thermophilic, eubacterial microorganism capable of growth at 70º-75ºC, was first isolated from a hot spring in Yellowstone National Park and the enzyme was characterized by Chein *et al*. (1976). The *Taq* polymerase activity more or less doubles from 65°C to 72°C and is optimal over a fairly broad pH range from 8.2-9.0 in 10 mM Tris. Alternative thermostable DNA polymerases have also been characterized and include: *T. thermophilus* DNA polymerase, *Bacillus stereothermophilus* and a DNA polymerase from the extreme thermophile *Thermococcus litoralis* (Rolfs *et al*.,1992).

4.2.2.2. Primers

Primers are specific oligonucleotides, designed to be complementary to the two 3'-ends of the specific DNA segment to be amplified. The sequence and the combination of the primers more than anything else determine the success or failure of an amplification reaction. Depending on their purpose, useful primers lengths are 14 to 40 bases long, although low complexity DNA (e.g. plasmids or previously amplified DNA) can be amplified with shorter primers (Saiki, 1989a, Rolfs *et al*., 1992). PCR primer selection programs are available and provide help with primer design and the calculation of annealing temperatures. However, the following guidelines can be useful when designing primers.

1. Primers should lie within highly conserved regions of the genome of the analysed species.
2. 3' ends of primers should be designed to code for the nucleotide sequences specifying conserved amino acids with nondegenerate codons (Trp, Met).
3. In order to prevent "primer dimers" and resultant waste of primers in the PCR, 3' ends of primers should avoid complementarity and should not end in palindromes.
4. Imbalanced distribution of G/C and A/T-rich domains should be avoided.

Bases that do not hybridise to the template may be added at the 5'end of a primer for introducing restriction sites or promotor sequences into the amplification product (Rolfs *et al*., 1992).

4.2.2.3. PCR Reaction Buffer

A PCR reaction mix includes the DNA sample to be amplified, reaction buffer, deoxyribonucleotide triphosphates, a pair of primers specific for the target sequence, and the thermostable Taq DNA polymerase. The reaction buffer for PCR with Taq polymerase typically contains 50 mM KCl, 10 mM Tris (pH 8.4), 1.5 mM $MgCl_2$ and 100 µg/ml gelatin. Changes to the PCR reaction buffer will usually effect the outcome of the amplification. Both KCl and Tris concentration can generally be lowered or increased without major effect, however, the concentration of $MgCl_2$ can have a profound effect on the specificity and yield of an amplification (Saiki, 1989b).

The $MgCl_2$ concentration may vary from approximately 0.5 mM to 5 mM to find the optimum. The concentration of free Mg^{2+} depends on [dNTP], [PP_i] and [EDTA]. Each of these compounds binds stoichiometrically with Mg^{2+} via the negative charges on the phosphate groups. Mg^{2+} influences enzyme activity, increases T_m of double-stranded DNA, and forms soluble complexes with dNTPs, which is essential for dNTP incorporation (Rolfs, 1992). Steitz (1998) suggests that DNA polymerases use a “two-metal-ion“ mechanism to catalyse nucleotide addition during elongation of a growing polynucleotide chain. The incoming nucleotide has two Mg^{2+} ions coordinated to its phosphate groups, and these metal ions interact with two aspartate residues that are highly conserved in DNA polymerases. Together, the two metal ions stabilize the penta-covalent transition state on the α phosphorus atom (Fig.4.5).

The deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP) are usually present at 50 to 200 µM of each. Higher concentrations may tend to promote misincorporations by the polymerase. These dNTPs can be acquired in either a freeze-dried form or as a neutralized aqueous solution and are stable at -20°C for some months in either constitution. As dNTPs appear to quantitively bind Mg^{2+}, the amount of dNTPs present in a reaction will determine the amount of free magnesium available. Consequently, if the dNTP concentration is changed significantly, a compensatory change in $MgCl_2$ may be necessary (Saiki, 1989a).

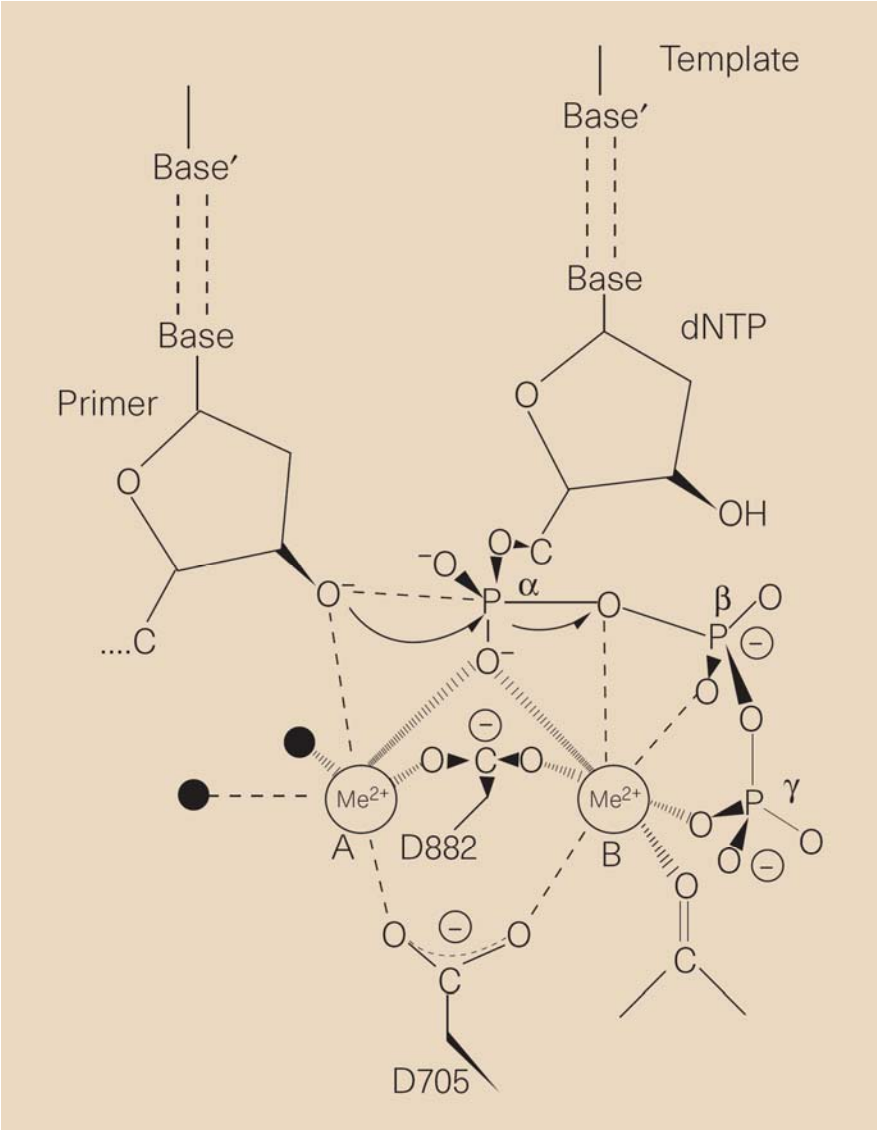


Figure 4.5: A mechanism for polymerase (Steitz,1998).

4.2.2.4. Target DNA

Numerous different sources can provide material for the successful amplification of DNA. The main requirements are that the DNA should be intact over the length which is to be amplified, and that inhibitors such as detergents, EDTA and traces of phenol are not present (Taylor 1991).

4.2.3. Reaction conditions

The selection of times, temperature and number of cycles are dependant on the DNA being amplified and the primers chosen. Reaction volumes normally vary between ten and 100 μl, although small volumes are an advantage, because of savings in reagent

costs when large numbers of samples need to be screened. In contrast, it may be technically easier to work to a slightly larger reaction volume (25-50 μl), when only a few samples are being processed (Taylor 1991).

Incubation times should be kept as short as possible to reduce the overall cycling time, to minimize denaturation of the *Taq* polymerase, and to minimize the risk of non-specific amplification. The number of cycles required depends on the abundance of target DNA and the efficiency of the PCR. These temperatures and the duration of each step are attained using a thermocycling machine, which is simply a heating block that can be programmed to alter its temperature and holding time for each step.

4.2.4. Detection of the reaction product

Once the PCR product is produced, it can be detected by various means. A general method is to analyse the product using agarose gel electrophoresis. During electrophoresis the DNA products are separated according to size, and DNA is stained with ethidium bromide before visualising under ultraviolet illumination. The product of a PCR should be one or more fragments of DNA of distinct length and should appear as sharp bands at expected size, although primers and small DNA products (primer-dimers) can be seen as rather diffused bands close to the front of the gel. Additional bands may be due to non-specific priming (Taylor 1991).

4.2.5. PCR in genetic diagnosis

The PCR has revolutionized the way genetic diagnosis can be performed. Previously, the diagnosis of many genetic diseases involved taking 10-20 ml blood sample followed by a lengthy protocol over a minimum of four or five days to produce an autoradiograph displaying the results. With the PCR it is possible to perform the same test in less than 10 h starting from samples as innocuous as a mouthwash or a pinprick blood spot.

To overcome some of the limitations of microscopy for detection of malaria, PCR-based assays have been developed for the detection and identification of malarial parasites. These methods have proved to be more specific and sensitive than conventional microscopy and some are reported to be able to detect as few as one parasite per μl of blood. PCR tests are also used in analysing mosquitoes and historical blood smears. Furthermore, the use of PCR technology does not require specific training for the

diagnosis of malaria, as the technology is broadly applicable to the detection of a wide variety of pathogens.

However, these assays are more expensive than microscopy and for diagnosis of a small number of samples are not as rapid as microscopy. PCR-based methods will therefore probably not replace microscopy for routine diagnosis of malaria in malaria-endemic countries, but are extremely valuable for epidemiological studies (Hänscheid & Grobusch 2002).

Many PCR-based assays have been reported for the diagnosis of malaria (Barker 1992, Brown 1992, Kain 1993, Snounou *et al*.,1993, Arai 1994). Snounou *et al*. (1993) were the first to describe a PCR-based assay for detection of all four species of human malaria. Their target was the gene coding for small subunit ribosomal RNA, and four sets of species-specific primers in four separate PCR reactions were required for their assay. The PCR-based assay used in this study makes use of genus-conserved primers, described by Li *et al.*, 1997, and also amplifies the ribosomal RNA (rRNA) genes. The target for PCR based assays i.e. the *Plasmodium* genome, and in particular the rRNA genes, will be discussed next.

4.2.6. *Plasmodium* genome

In 1996 an international effort was launched to sequence the *Plasmodium falciparum* genome with the expectation that the genome sequence would open new avenues for research on drug therapy, vaccines and diagnosis for malaria. In 2002 Gardner *et al.* reported an analysis of the genome sequence of *P. falciparum* clone 3D7 (Garner 2002). The genome sequence of the model rodent malaria parasite, *P. yoelii yoelii* has also been described (Carlton 2002), however, only *P. falciparum* will be discussed in this section.

The *P. falciparum* 3D7 nuclear genome consists of 14 chromosomes and is composed of 22.8 megabases (Mb). These chromosomes range in size from approximately 0.643 to 3.29 Mb. The overall (A+T) composition is 80.6% and rises to ~90% in introns and intergenic regions. Approximately 5,300 protein-encoding genes were identified, suggesting an average gene density of one gene per 4,338 base pairs.

This *Plasmodium* also contains a mitochondrial genome and an apicoplast. The mitochondrial genome is small and only consists of about 6 kb. The apicoplast is a relict plastid, harboured by malaria parasites and other members of the Phylum Apicomplexa, and is homologous to the chloroplast of plants and algae. The apicoplast is essential for survival, but its role is unclear. The 35-kb apicoplast genome encodes only 30 proteins, but as in mitochondria and chloroplasts, the apicoplast proteome is supplemented by proteins encoded in the nuclear genome and post-transcriptionally targeted into the organelle (Gardner *et al*., 2002).

4.2.6.1. Ribosomal RNAs

The study of rRNAs has attracted the attention of biologists for the reason that these molecules are central to defining the catalytic nature of the ribosome. Ribosomal RNAs have virtually the same structure and function in all organisms and the gene families that encode these molecules display similar organizational structure and transcriptional control (Waters 1994, McCutchan 1995).

Eukaryotes typically contain long tandemly repeated arrays of rRNA genes. However, *Plasmodium* parasites contain several single rRNA units distributed on different chromosomes (Gardner 2002). The single rRNA unit is arranged in a standard fashion, with a copy of the small subunit (SSU) or 18S rRNA, an internal transcribed spacer (ITS1), the 5.8S rRNA, another internal transcribed spacer (ITS2) and the large subunit (LSU) or 28S rRNA genes proceeding in a 5' to 3' direction. The sequence of an rRNA gene in one unit differs from the sequence of the corresponding rRNA in the other units. The expression of these rRNA units is developmentally controlled, resulting in the expression of a different set of rRNAs at different stages of the parasite life cycle (Gunderson *et al*., 1987). By changing the properties of its ribosomes the parasite is able to alter the rate of translation, thereby changing the rate of cell growth or altering patterns of cell development (McCutchan 1995, Gardner 2002, Mercereau-Puijalon *et al*., 2002).

There are three types of rRNA genes, the A-type, the S-type and the O-type, each expressed at different stages during the development of the parasite (Figure 4.6). The A-type rRNA is the dominant transcript in erythrocytic stages including gametocytes, the initial form of sexual stages. After zygote formation in the mosquito midgut, the O-type rRNA replaces the A-type. Transcription of the O-type rRNA gene continues in the

oocyst through the development in the mosquito. As soon as the differentiation of sporozoites begins in the maturing oocysts (about a week after the infectious blood meal), the S-type rRNA is initiated. The S-type rRNA corresponds with the period for differentiation of sporozoites, which can migrate from mature oocyst to the salivary gland of the mosquito, ready for infection of humans. The A-type rRNA replaces the S-type rRNA when merozoites differentiate in maturing liver stage schizonts. The merozoites released from the mature schizonts will initiate the erythrocytic stage of the parasites in which the A gene dominates (McCutchan *et al*., 1995, Li *et al.*, 1997, Waters *et al*., 1997).

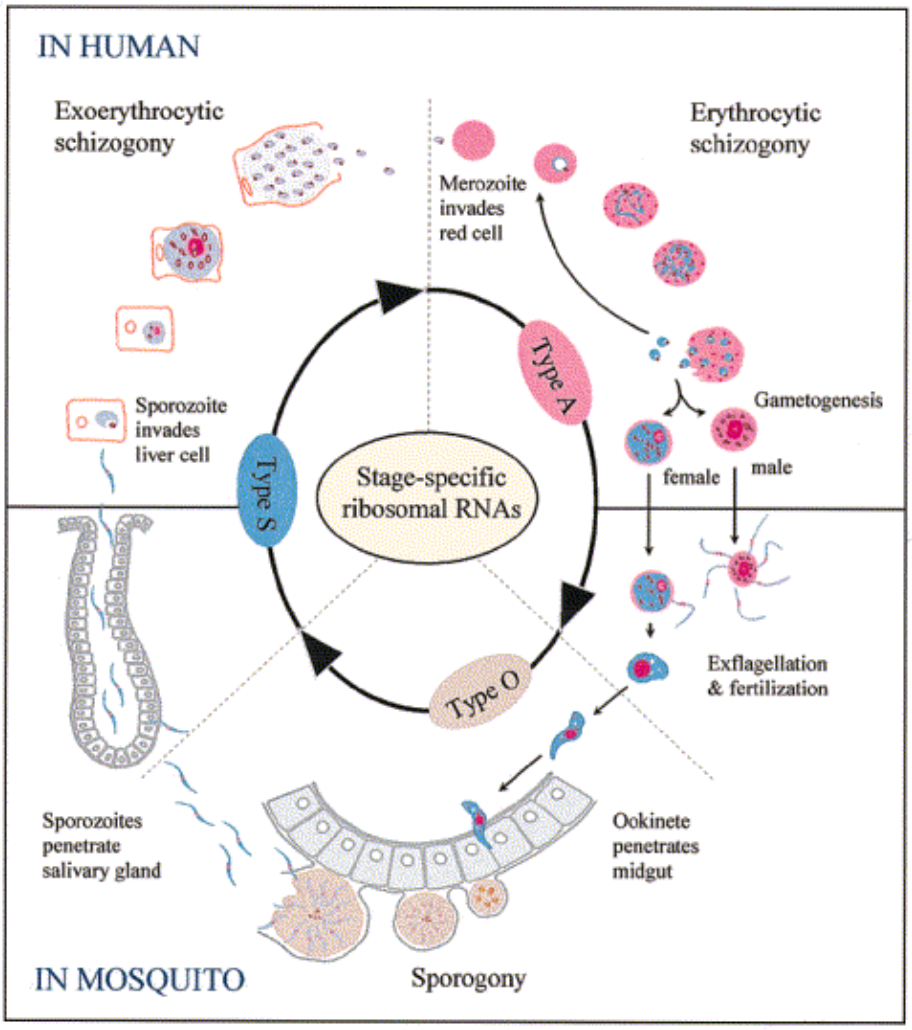


Figure 4.6: A summary of the expression of *Plasmodium* SSU rRNA genes (McCutchan *et al*., 1995).

Ribosomal RNA, particularly the SSU rRNA, is an ideal molecular target for malaria parasite identification. The sequence of the SSU rRNA is composed of a mixture of conserved and variable regions, allowing the amplification of sequences from samples

using primers that are conserved within every member of the genus *Plasmodium*. The primers are usually located in areas bounding conserved regions and species-specific variable regions. The genus-conserved primer pair 841-844 used in this study was published by Li *et al*. (1995), and covers the most variable regions (V7 and V8) of the SSU rRNA (see figure 4.7). This ensures the differentiation of both the genes from different species and the different types of gene from the same species. The primer combination also includes an internal genus-conserved sequence located between V7 and V8, which can serve to confirm the identification of the sequence as *Plasmodium*-related (Li *et al.*, 1995).

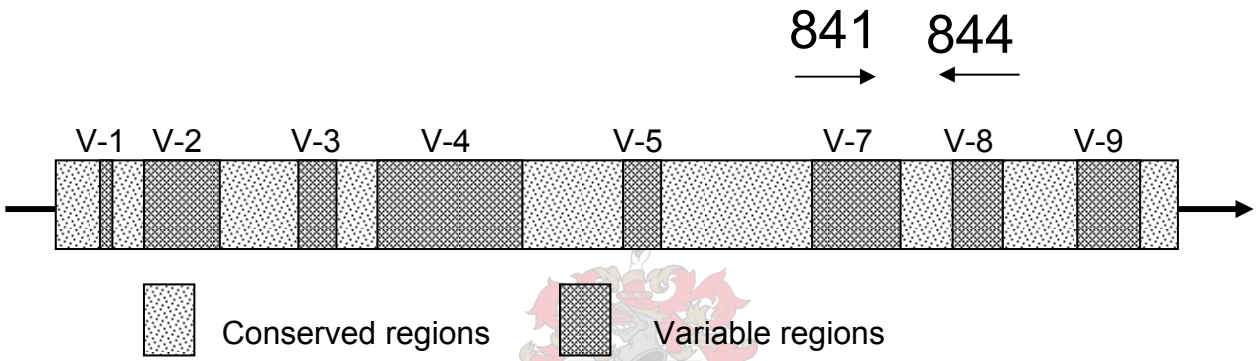


Figure 4.7: A schematic representation of conserved and variable regions for *Plasmodium* SSU rRNAs based on available sequences. The variable regions differ in both size and nucleotide composition (Li *et al*., 1995).

Immunological and PCR-based methods are very powerful tools in epidemiological studies of avian malaria in the African Penguin, especially when used in combination. The ELISA for the detection of antibody levels to avian malaria is sensitive and capable of indicating whether the penguin had contact with avian malaria, however, rising antibody levels could not be used to distinguish whether penguins arrived at the SANCCOB facility with an existing malaria infection or whether they were newly infected or suffered from recrudescence after admission in the facility. However, the PCR-assay is capable of distinguishing between existing malaria infections as opposed to new infections and recrudescence, and is extremely sensitive. For that reason, investigation into avian malaria at the SANCCOB facility and breeding colonies of the African Penguin, utilised ELISA and PCR assay discussed above. The results obtained are presented in the next two chapters.

CHAPTER 5

INVESTIGATIONS INTO AVIAN MALARIA IN THE AFRICAN PENGUIN DURING REHABILITATION

5.1. Introduction

The Southern African Foundation for the Conservation of Coastal Birds is the main facility at which oiled and injured seabirds are treated in the Western Cape Province, South Africa. SANCCOB is mainly dedicated to the de-oiling and rehabilitation of weakened penguins, although other sick or injured seabirds are also rehabilitated at the center.

Penguins arrive at the center in different ways. Members of the public or SANCCOB volunteers mostly collect single birds, while birds from breeding colonies are usually collected by staff maintaining the colonies (Robben Island Museum, South African National Parks and Western Cape Nature Conservation Board) and organizations working in the colonies (Marine and Coastal Management and Avian Demography Unit, UCT). The most frequent reason for admission is oiling. The majority of oiling incidents occur during winter storms between the months of July and September. This falls in the latter part of the main breeding season when adults are feeding large chicks and have to travel far to find sufficient food. Prevailing northwesterly winds and currents are also responsible for carrying oil onshore. Other reasons for admission include weakness, wounds, injuries, arrested moult and abandoned chicks. The abandoned chicks mainly come from breeding colonies at Robben Island, Boulders and Dyer Island where they are abandoned primarily during November and December when parents enter moult (changing feathers). After rehabilitation healthy birds are released from a boat close offshore of the penguin colony at Robben Island.

During rehabilitation a blood sample is evaluated by blood smear once a week in order to screen for malaria and other parasites. A blood sample is also evaluated on admittance to the center. Penguins, which are infected with malaria, receive anti-malaria treatment (normally a 10-day treatment) consisting of both primaquine and chloroquine (Darmal®). Since the drug can damage the liver, it is also supplemented

with Essentiale®, a vitamin supplement for maintaining optimum liver function. Blood smears have to be negative for parasites before the penguin can be released.

There are three ways in which penguins at SANCCOB can become infected with malaria. Firstly, penguins may contract the disease in the facility via mosquitoes. SANCCOB is situated next to Rietvlei, a large shallow freshwater lake with abundant bird life, both aquatic and on land, and a large number of culicine mosquitoes. SANCCOB is therefore close to potential malaria reservoirs and vectors. Penguins may also harbour malaria parasites on arrival and may suffer from parasite recrudescence if weakened due to oiling, injury or stress. Lastly, a high percentage of birds are being admitted with parasitaemia, indicating that they have contracted malaria elsewhere and possibly in the breeding colony (Parsons & Underhill 2004).

Three *Plasmodium* species, namely *Plasmodium juxtanucleare*, *P. relictum* and *P. elongatum*, have been identified to cause infection in the African Penguin. *P. relictum* and *P. elongatum* is well known for occurring naturally in penguin breeding colonies (Bennett *et al*., 1993). *P. juxtanucleare,* however, has only recently been identified to cause mortalities in African Penguins at SANCCOB (Grim *et al*., 2003). It was also the first identification of this species as a pathogen in non-gallinaceous birds. *P. juxtanucleare* occur naturally in Greywing Francolins (Earle *et al*., 1991), a bird commonly found at Rietvlei and around the SANCCOB facility and can therefore possibly act as a reservoir for avian malaria.

SANCCOB is currently monitoring parasitemia in penguins using thin blood smears. This is, however, as discussed in chapter 4, an inadequate method for diagnosing avian malaria. For this reason Botes (2004), developed an ELISA based on the cross-reactivity between *P. relictum, P. elongatum* and *P. falciparum* antibodies, for the detection of anti-*Plasmodium* antibodies. This ELISA was based on a similar ELISA developed by Graczyk *et. al*. (1993). The assay was used to detect antibody levels upon entry and during rehabilitation at SANCCOB from October 2001 to Jan 2003 with the aim of investigating whether oiling reduced the immunity of penguins and whether infection due to the position of the facility is exacerbating the problem (Botes 2004).

The objective of this study was first to continue the determination of antibody levels to avian malaria of penguins entering the facility, in order to allow an evaluation of the antibody levels to avian malaria for two full calendar years. This included determining

whether the ability of penguins to produce an anti-*Plasmodium* antibody response influences their survival rate; and whether oiling influences the penguin's ability to produce an anti-*Plasmodium* immune response. Second, infection of penguins during rehabilitation was investigated using a PCR assay for the detection of all malaria infections. The PCR results were used to determine whether penguins were infected with malaria when they arrive at SANCCOB or whether they become infected during rehabilitation or suffer from recrudescence. Lastly, samples taken from Greywing Francolins caught in close proximity to SANCCOB were included in the PCR analysis, in order to determine if they could serve as a possible avian malaria reservoir.

5.2. Materials and Methods

5.2.1. Sample collection

Blood samples were collected from the SANCCOB facility on a weekly basis. Blood was collected by heparinized syringe venipuncture. Blood (80 μl) was collected in calibrated heparinized capillary tubes, deposited on filter paper (Immunovet Services) and air-dried. These samples were taken from a total of 30 penguins (unless fewer birds were actually being rehabilitated) on entry to the facility and for up to four weeks after submission. Samples were collected along with routine blood samples taken weekly for malaria blood smears in order to reduce additional work and stress to penguins during the rehabilitation process. Healthy birds were released, even though they were on trial, in order to prevent additional costs to the facility. The released birds were replaced in an attempt to retain 30 samples per week.

Prior to ELISA testing, blood elements were eluted as hemosylate from the filters by incubating them overnight at 4ºC in 333.33 μl of distilled water containing 0.4% of Tween 20. Sera with high anti-*Plasmodium* antibody levels were obtained from penguins being rehabilitated at SANCCOB and used as positive controls. A serum sample obtained from a penguin chick that was artificially hatched in isolation and kept indoors to exclude mosquitoes, was used as a negative control.

5.2.2. ELISA Capture Antigen

The capture antigen consisted of a 16 amino acid peptide, with the sequence $(NANP)_4$, which corresponds to the repeat region of the CS-protein of *P. falciparum* (Ballou *et al.*, 1985) and was synthesized by the BIOPEP Peptide Synthesis Laboratory at the Biochemistry Department, University of Stellenbosch. Prior to use, the peptide was conjugated to BSA in a 3:1 ratio of malaria peptide to BSA (Botes, 2004).

5.2.3. Rabbit anti-penguin Ig antibodies

The rabbit anti-penguin Ig antibodies were previously prepared by Botes (2004). Briefly, blood was drawn from penguins housed at the SANCCOB rehabilitation facility and serum isolated. Penguin immunoglobulin (Ig) was purified from the serum using 50% ammonium sulphate precipitation. The isolated penguin Ig was absorbed to acid-treated naked bacteria and used for the immunization of rabbits (Bellstedt *et al*., 1986). High-titre anti-penguin immunoglobulin antibodies were raised in this way.

5.2.4. Biotinylation of rabbit anti-penguin antibodies

The Ig fraction of the rabbit anti-penguin Ig serum was isolated as follows. The rabbit serum was diluted (1:2) with phosphate buffered saline (PBS, 0.15 M, pH 7.2) and Ig precipitated by the addition of an equal volume of saturated ammonium sulphate solution at 4°C. The solution was centrifuged at 27 200 x g for 20 min, the supernatant removed and the precipitate was dissolved in PBS. The precipitation step was then repeated and the final precipitate was dissolved in an amount of PBS equivalent to the original serum volume. The solution was dialyzed for approximately 16 h at 4°C against carbonate buffer (0.1 M, pH 8.3). The buffer was changed once during this time. The Ig concentration in the final dialysate was determined by absorption (280 nm) using BSA as standard. Carbonate buffer was then added in order to obtain an Ig concentration of 5 mg/ml. A 2 mg/ml solution of biotinamidocaproate N-hydroxysuccinimide ester (Biotin, Sigma) in N, N-dimethylformamide (DMF) was prepared. The solution was added in a 1:4 ratio (Biotin reagent : Ig solution) to the Ig solution while stirring slowly for 2 h at room temperature followed by dialysis against PBS buffer overnight at 4°C. Glycerol was finally added in a 1:1 ratio and the final preparation stored at -20°C. This procedure is routinely performed in this laboratory and biotinylation efficiency is

therefore not routinely checked. However, the subsequent binding of the Streptavidin to the biotinylated antibody in the ELISA, in comparison to negative controls, is a proof of the successful biotinylation of antibodies in this procedure.

5.2.5. ELISA for penguin anti-*Plasmodium* antibodies

Microtitre plates (96 well, Nunc, Medisorp, Denmark) were coated (100 μl/well) with 0.5 μg/ml of 3:1 ratio of malaria peptide-BSA conjugate in carbonate buffer (50 mM, pH 9.6) overnight at 4°C. The coating solution was decanted and the plate blocked with casein buffer (0.5% casein, 0.15 M NaCl, 0.01 M Tris-HCl, 0.02% thiomersal, pH 7.6, 200 μl/well) for 1 h at 37°C. The plates were subsequently washed three times using PBS-Tween. Samples were diluted with casein-tween (casein buffer containing 0.1% Tween 20). Blood eluted from filter paper was diluted 50x and serum samples 200x. Standard penguin sera with negative and high anti-*Plasmodium* antibody levels were included with every assay. Triplicate samples were pippeted added in wells (100 μl/well) and incubated for 3 h at 37°C. Plates were washed three times using PBS-Tween. Biotinylated rabbit anti-penguin antibody suitably diluted (1:100) in casein-Tween was subsequently added (100 μl/well) and incubated for 1 h at 37°C. Thereafter the plates were washed three times using PBS-Tween. This was followed by the addition of Streptavidin-horseradish-peroxidase conjugate (Zymed), diluted 1/100 in casein-Tween (100 μl/well) and incubated for 1 h at 37°C. The plates were washed three times with PBS-Tween. The substrate solution was added (0.05% Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 0.015% H_2O_2 in 0.1 M citrate buffer, pH 5, 100 μl/well) and incubated at 37°C for 30 min. Finally the absorbance was measured at 405 nm on a Titertek Multiskan spectrophotometer.

5.2.6. PCR analysis

Individual birds showing high as well as low antibody levels were chosen for PCR analysis. These samples were chosen so as to represent both summer and winter data. The DNA was isolated and the PCR were performed on the blood-soaked filter strip eluates used in the ELISA, from each weekly sample taken from the penguins during their stay at the facility.

5.2.6.1. DNA extraction

DNA was extracted using DNAzol BD (Gibco BRL, Baltimore, USA). The manufacturer's instructions were modified in order to extract DNA from dried blood samples on filter paper strips. The blood was eluted from the filterpaper using H_2O with 0.4% Tween 20 and subsequently lysed by mixing 100 μl of the blood eluate with 200 μl of DNAzol BD. DNA was precipitated from the resulting lysate with 80 μl of isopropanol. The mixture was stored at -20°C for 90 min and subsequently centrifuged at 13 000 x g for 10 min at 4°C. The supernatant was removed and the pellet washed with 100 μl DNAzol BD. The mixture was inverted and incubated at 37°C for 1 h in order to disperse the pellet. The mixture was then again centrifuged at 13 000 x g for 10 min at 4°C. After removing the supernatant, the pellet was washed once more by adding 200 μl of 75% ethanol and incubating at room temperature for 30 min. The mixture was again centrifuged at 13 000 x g for 10 min at 4°C. The ethanol was removed and the pellet air-dried at 37°C for about 30 min. After drying, the pellet was redissolved overnight in 25 μl TE buffer (10 mM Tris-HCl, pH 8.0, I mM EDTA) at 4°C.

5.2.6.2. PCR

The primers used to identify the SSU rRNA gene were sense 841 (5' – GAACGAGATCTTAACCTGC – 3') and anti-sense 844 (5' – TAITGATAAAGATTACCTA – 3') (I = inosine) as previously described by Li *et al*. (1995). The assay was performed using a 20 μl reaction volume containing 0.5 U Super-therm *Taq* polymerase (Southern Cross, Biotechnology); 2 μl of 10X PCR Buffer (Southern Cross, Biotechnology): 1.5 mM of $MgCl_2$ (Southern Cross Biotechnology); 20 pmoles of each primer; 200 μM of each nucleotide (dATP, dGTP, dCTP, dTTP); and 0.4 μl of the extracted DNA samples. Genomic DNA samples from *P. falciparum* and *P. gallinaceum* were used as positive controls and water was used as a negative controle.

PCR amplification was performed with a Hybaid thermal cycler using the following conditions: samples were subjected to 40 cycles of 94 °C for 30 s, 35 °C for 30 s and 72 °C for 30 s. These steps were followed by an additional primer extension step of 7 min at 72 °C. The PCR products were evaluated by electrophoresis on 2% agarose gels stained with ethidium bromide.

5.2.7. Francolin samples

Five Greywing Francolins were caught in close proximity to the SANCCOB facility. Blood samples were collected on filter paper strips as indicated in section 5.2.1. Blood elements were eluted as described in the same section. DNA was extracted and PCRs performed as described in sections 5.2.6.1 and 5.2.6.2 respectively. The PCR products were evaluated by electrophoresis on 2% agarose gels.

5.2.8. Data analysis

The release of apparently healthy birds was not delayed because they were on trial and this meant that the number of data points that were collected for each bird was not always the same. In order to obtain a meaningful picture of the anti-malaria antibody levels over a period, the data was combined in two-month periods and analysed from the date of entry to the facility. The date of entry was taken as week 1 and subsequent weeks of sampling within a specific two-month period were taken as weeks 2, 3 and 4 respectively. The average ELISA titre values for each week were plotted in each respective two-month period. However, because there were too many missing data points for the fourth week, only the data for the first three weeks was used for the statistical analysis. Statistical analysis of the data was performed by Willem Botes, using the General Linear Models (GLM) procedure in the Statistical Analysis System (SAS) Enterprise Guide (version 1.3.0.161). The data generated was additionally separated into: (a) birds that survived (and were released) and birds that died with a view to possibly establishing the role of immunity in mortalities at the facility: and (b) birds that were oiled as opposed to birds that were not oiled to possibly establishing the effect of oiling on the immunity at the facility.

5.3. Results

The antibody levels of the penguins that were released and those that died during rehabilitation were compared in figures 5.2 and 5.3. Statistical analysis of the data indicated that there was no significant difference between the immune response between the penguins that were released and those that died. The P-values on the individual graphs show the significant time responses. The antibody levels of the penguins that were oiled and those that were not oiled on admission to the facility were

compared in figures 5.4 and 5.5 with the P-values on the individual graphs showing the significant time responses. Statistical analysis indicated no significant difference between these two groups and oiling therefore does not have a significant effect on the ability to produce an anti-*Plasmodium* immune response.

From the ELISA results it was observed that the anti-*Plasmodium* immune response is higher in the warmer spring and summer time (October – March) than in the colder autumn, winter and early spring months (April – September). This phenomenon coincided with the number of birds that was diagnosed malaria positive by blood smear: 18% - 26% in the spring and summer, and 6 – 13% in autumn and winter. Mortalities due to malaria were also higher in the summer (5% - 11%) than in winter (0% - 2%).

Individual birds showing high and low ELISA titre values were chosen for PCR analysis. Penguins with high antibody levels were expected to have been infected with malaria, whereas penguins with low antibody levels were expected not to have been infected. The DNA was isolated and PCRs performed for each week that the penguin stayed at the facility in order to determine whether the penguin was infected with *Plasmodium* on arrival, became infected at the facility or whether it was due to recrudescence. The following assumptions were made in regard to the manner of infection: if the penguin tested positive for malaria in the first week, it arrived at the facility already infected; and if the penguin tested negative for malaria in the first week and tested positive in any of the following weeks, the penguin was either infected at the facility or suffered from recrudescence. It was not possible to distinguish between whether the penguin was infected at the facility or suffered from recrudescence.

The PCR products were evaluated by electrophoresis on 2% agarose gels stained with ethidium bromide. The agarose gels were photographed under ultravioletlight and are shown in figures 5.6, 5.7, 5.8, 5.9, 5.10 and 5.11. In all cases the positive control, either *P. gallinaceum* or *P. falciparum* gave a positive result and the negative control was always negative.

Positive samples can have more than one band since the primer pair flanks different types of SSU rRNAs. These different types of SSU rRNA genes are each expressed at different stages during the development of the parasite and the sequence of an SSU rRNA gene in one unit differs from the sequence of the corresponding SSU rRNA in the other unit. The rRNA genes of the *Plasmodium* genome have been discussed in detail

in section 4.2.6.1. Figure 5.1 shows an agarose gel with the vastly different PCR products from SANCCOB penguins, penguins from breeding colonies, and the genomic *Plasmodium* DNA used as positive controls (*P. gallinaceum* and *P. falciparum*). We observed in general either 650 bp or 1200 bp amplification products, or both (see figure 5.1, lanes 1-4). In some cases, in addition to 650 bp and 1200 bp, we also observed amplification products of 300 bp and 400 bp (lane 5 and 7, figure 5.1). Lanes 3, 4 and 7 were negative.

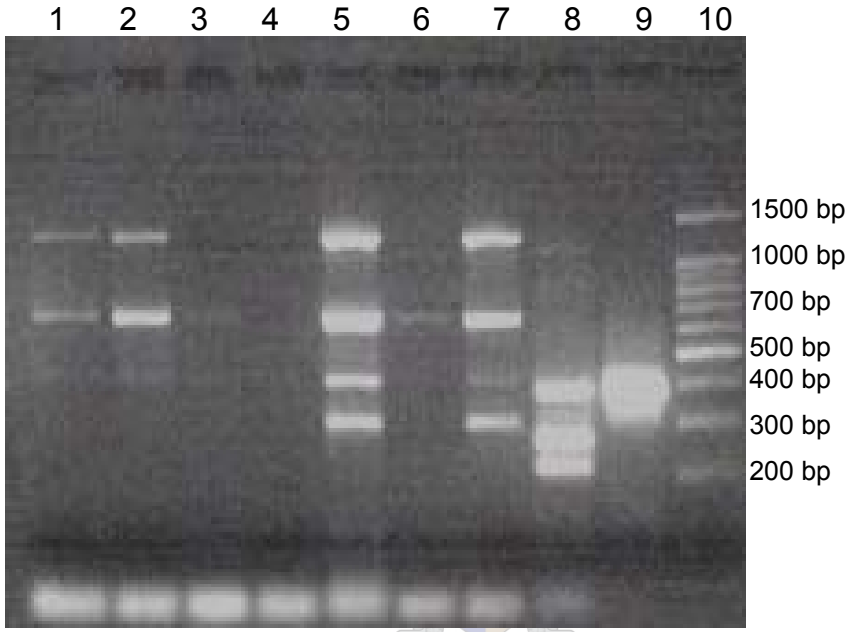
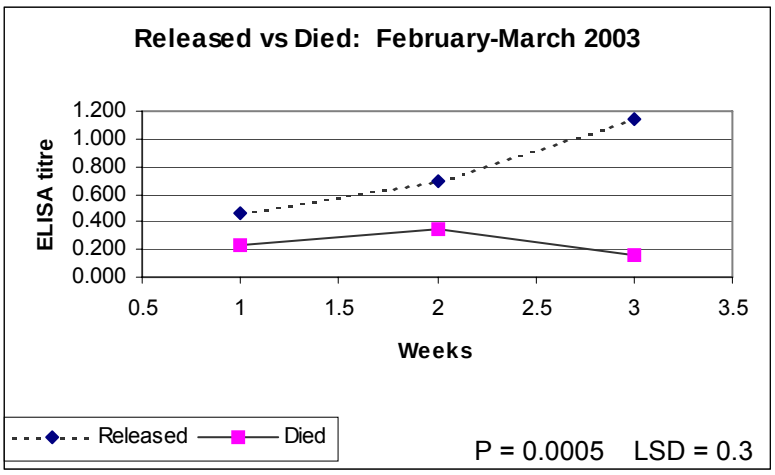


Figure 5.1: Comparison of PCR products from samples taken from penguins at SANCCOB and the breeding colonies: Lane 1: Penguin 284; Lane 2: Penguin 285; (Both from SANCCOB) Lane 3: Robben Island; Lane 4: Betty's Bay; Lane 5: Dassen Island; Lane 6: P. gallinaceaum; Lane 7: P. falciparum; Lane 8: 100bp DNA ladder.

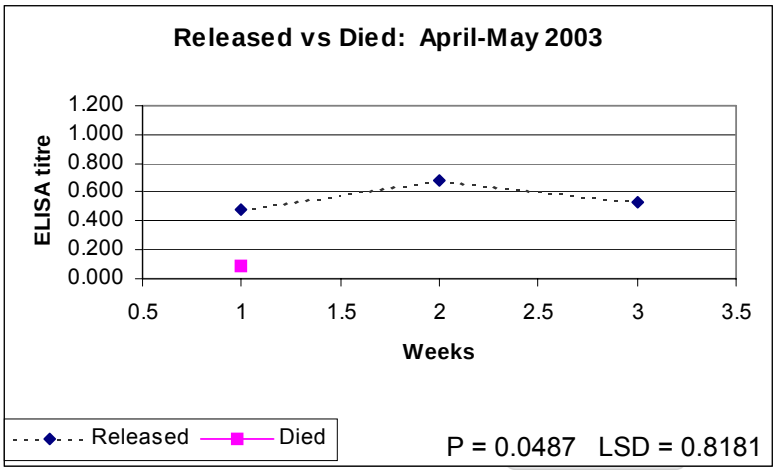
One possible explanation for the difference in size of the PCR products may be that the parasites were in different developmental stages when the DNA was extracted (see later for example). Therefore it is possible that more than one rRNA gene type was amplified. However, the difference between PCR results may also indicate infection with different *Plasmodium* species and possibly more than one in some penguins (i.e. lane 3 and 4, figure 5.1). The difference between the amplification of *P. gallinaceum* and *P. falciparum* indicates that the different species of *Plasmodium* reveals different results. Furthermore, the *Plasmodium* species found in the African penguins differed substantially from *P. gallinaceum* and *P. falciparum* in their PCR products (compare lanes 8,9 with 1,2,3,5 in figure 5.1).

In figure 5.5, penguin 047 is a clear example of RNA type switching from week 1 (650 bp amplification product) to week 2 (1200 bp product). A possible explanation can be that the parasite was in different developmental stages when the samples were taken. Penguin 068 shows multiple bands during the fourth week that was not present during the first three weeks. It might be that the penguin got infected with a second *Plasmodium* species before the sample for the fourth week was taken. Penguin 074 shows two bands (650 bp and 1200 bp) during the first and the second week, indicating that the developmental stage of the parasite were possibly not synchronised.

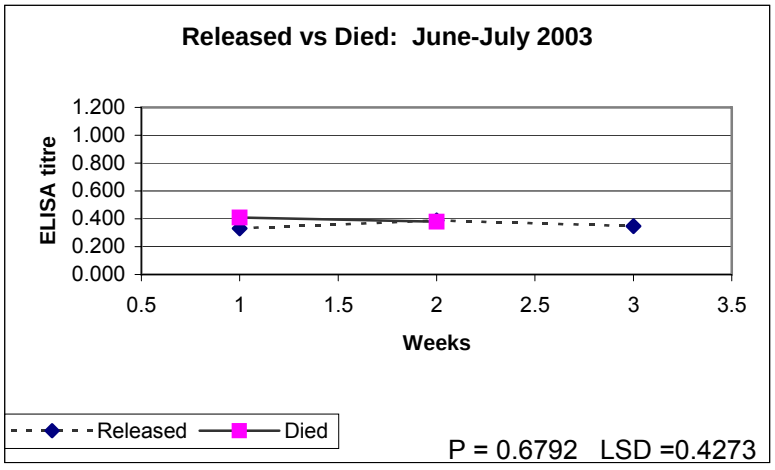
The PCR results obtained using DNA samples isolated from penguin blood, were separated into summer and winter and the following information is given with the PCR results for each individual bird: the ELISA titre value obtained for each sample; whether the penguin was released or died; whether it tested positive for malaria by blood smear (and treated prophylactically) at any time during its stay at the facility; and whether or not it was oiled when admitted to the facility. From the PCR results it was observed that penguins not only contract malaria at the SANCCOB facility, but also arrive infected. Almost half of the 30 birds chosen for PCR analysis were positive on arrival indicating that they contracted the parasite prior to admittance to the facility. Four of the birds did not contract malaria during any time at the facility and the rest either suffered from recrudescence or were infected at SANCCOB.



	Penguin samples from SANCCOB	Blood smear positive
Total	37	7 (19%)
Released	27	3
Died	10	4
Malaria deaths	2 (5%)	2

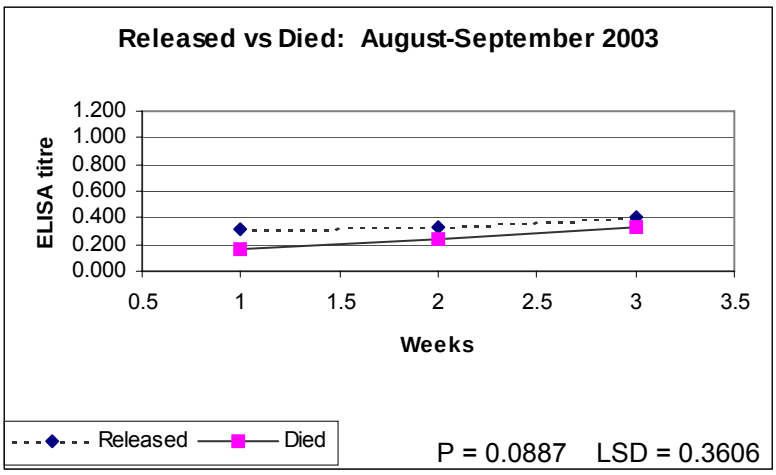


	Penguin samples from SANCCOB	Blood smear positive
Total	53	7(13%)
Released	50	6
Died	3	1
Malaria deaths	1 (2%)	1

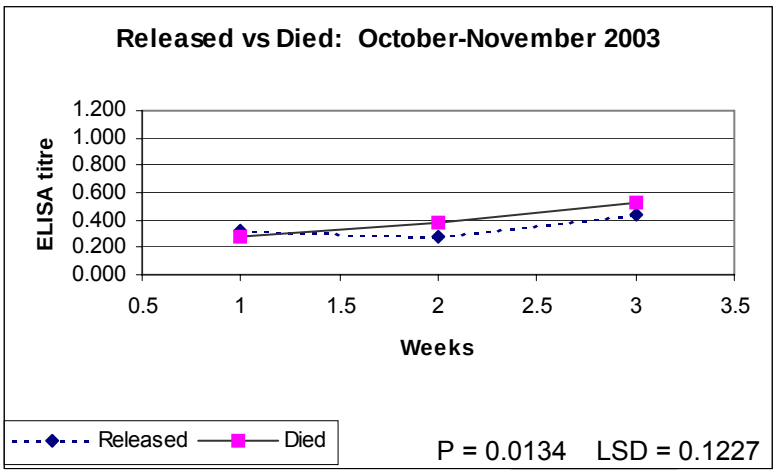


	Penguin samples from SANCCOB	Blood smear positive
Total	70	6(8%)
Released	68	6
Died	2	0
Malaria deaths	0	0

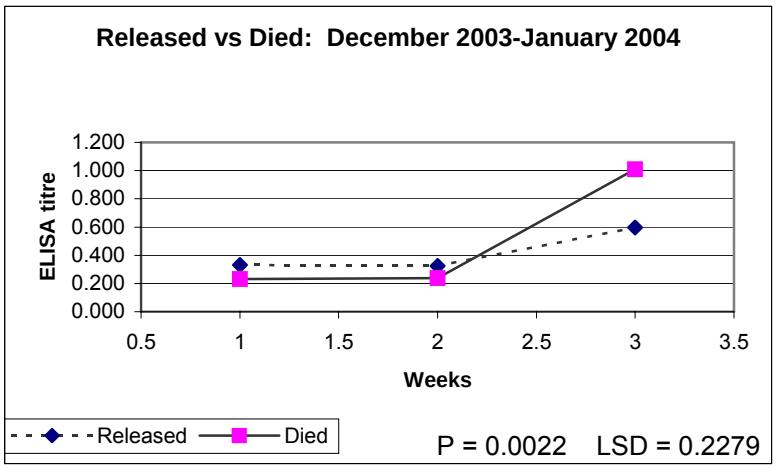
Figure 5.2: The antibody response of penguins that were released versus the penguins that died during rehabilitation from February to July 2003.



	Penguin samples from SANCCOB	Blood smear positive
Total	82	5(6%)
Released	80	5
Died	2	0
Malaria deaths	0	0

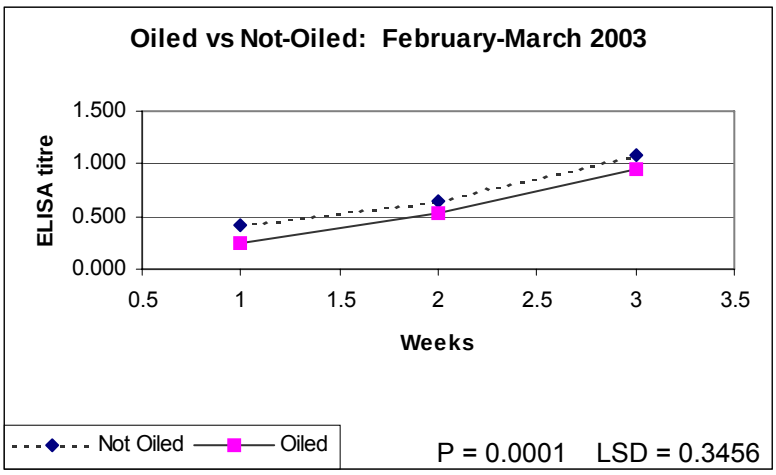


	Penguin samples from SANCCOB	Blood smear positive
Total	64	17(26%)
Released	53	11
Died	11	6
Malaria deaths	7 (10%)	6

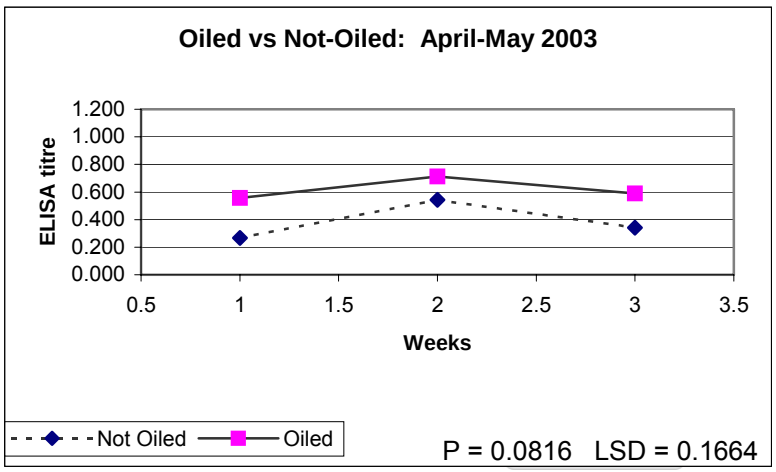


	Penguin samples from SANCCOB	Blood smear positive
Total	54	12(22%)
Released	42	6
Died	12	6
Malaria deaths	6(11%)	6

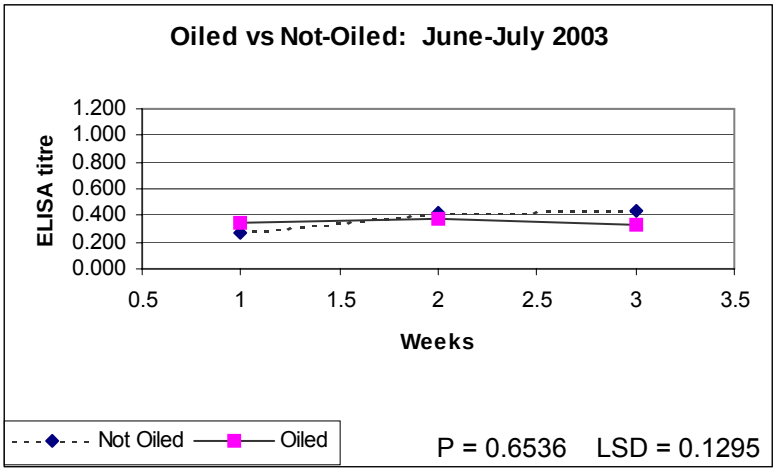
Figure 5.3: The antibody response of penguins that were released versus the penguins that died during rehabilitation from August 2003 to January 2004.



	Penguin samples from SANCCOB	Blood smear positive
Total	37	7
Oiled	5	1
Not-Oiled	32	6
Malaria deaths (oiled penguins only)		1

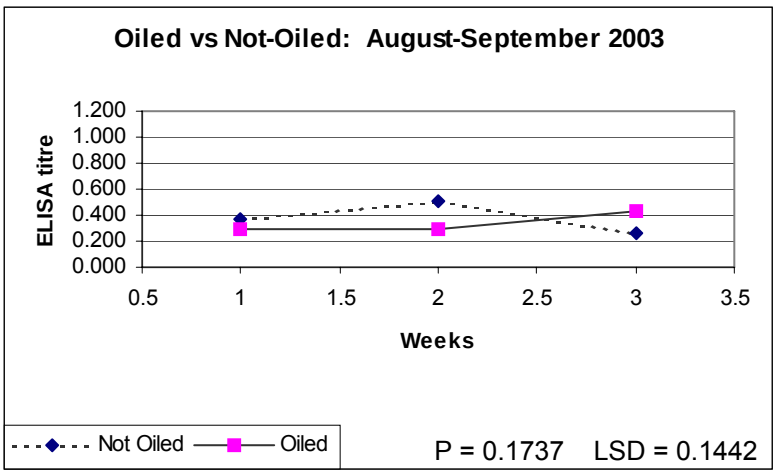


	Penguin samples from SANCCOB	Blood smear positive
Total	53	7
Oiled	35	2
Not-Oiled	18	5
Malaria deaths (oiled penguins only)		0

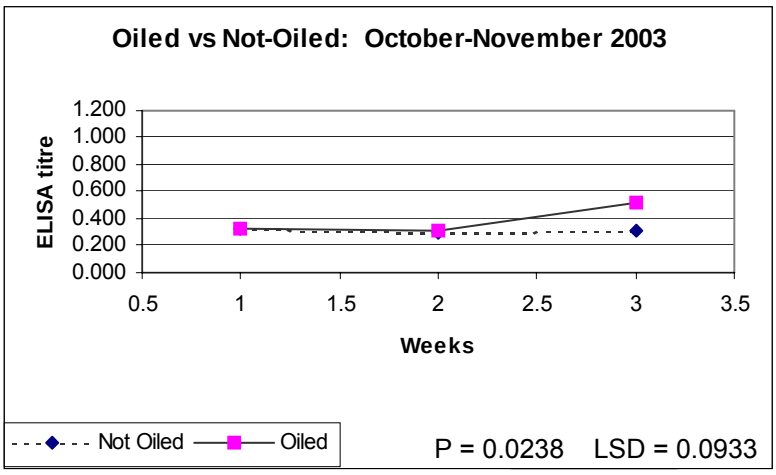


	Penguin samples from SANCCOB	Blood smear positive
Total	70	6
Oiled	55	2
Not-Oiled	15	4
Malaria deaths (oiled penguins only)		0

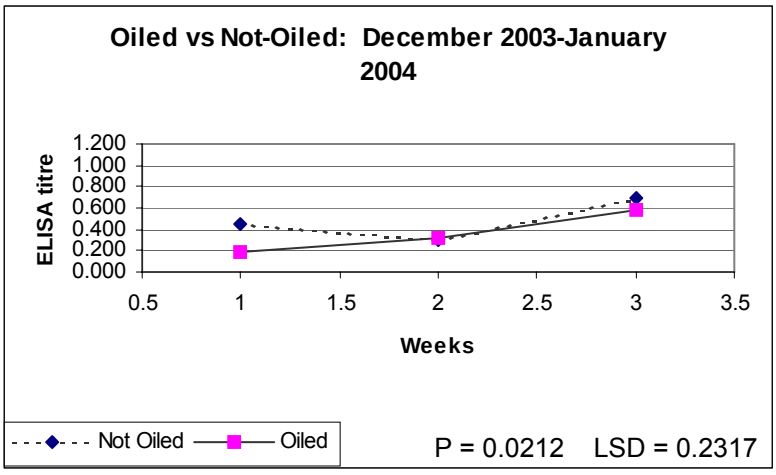
Figure 5.4: The antibody response of penguins that were oiled versus the penguins that were not oiled on admittance to the rehabilitation centre during February to July 2003.



	Penguin samples from SANCCOB	Blood smear positive
Total	82	5
Oiled	70	4
Not-Oiled	12	1
Malaria deaths (oiled penguins only)		0



	Penguin samples from SANCCOB	Blood smear positive
Total	64	17
Oiled	40	7
Not-Oiled	24	10
Malaria deaths (oiled penguins only)		2



	Penguin samples from SANCCOB	Blood smear positive
Total	54	12
Oiled	28	6
Not-Oiled	26	6
Malaria deaths (oiled penguins only)		4

Figure 5.5: The antibody response of penguins that were oiled versus the penguins that were not oiled on admittance to the rehabilitation centre during February to July 2003.

SUMMER (NOVEMBER – APRIL)

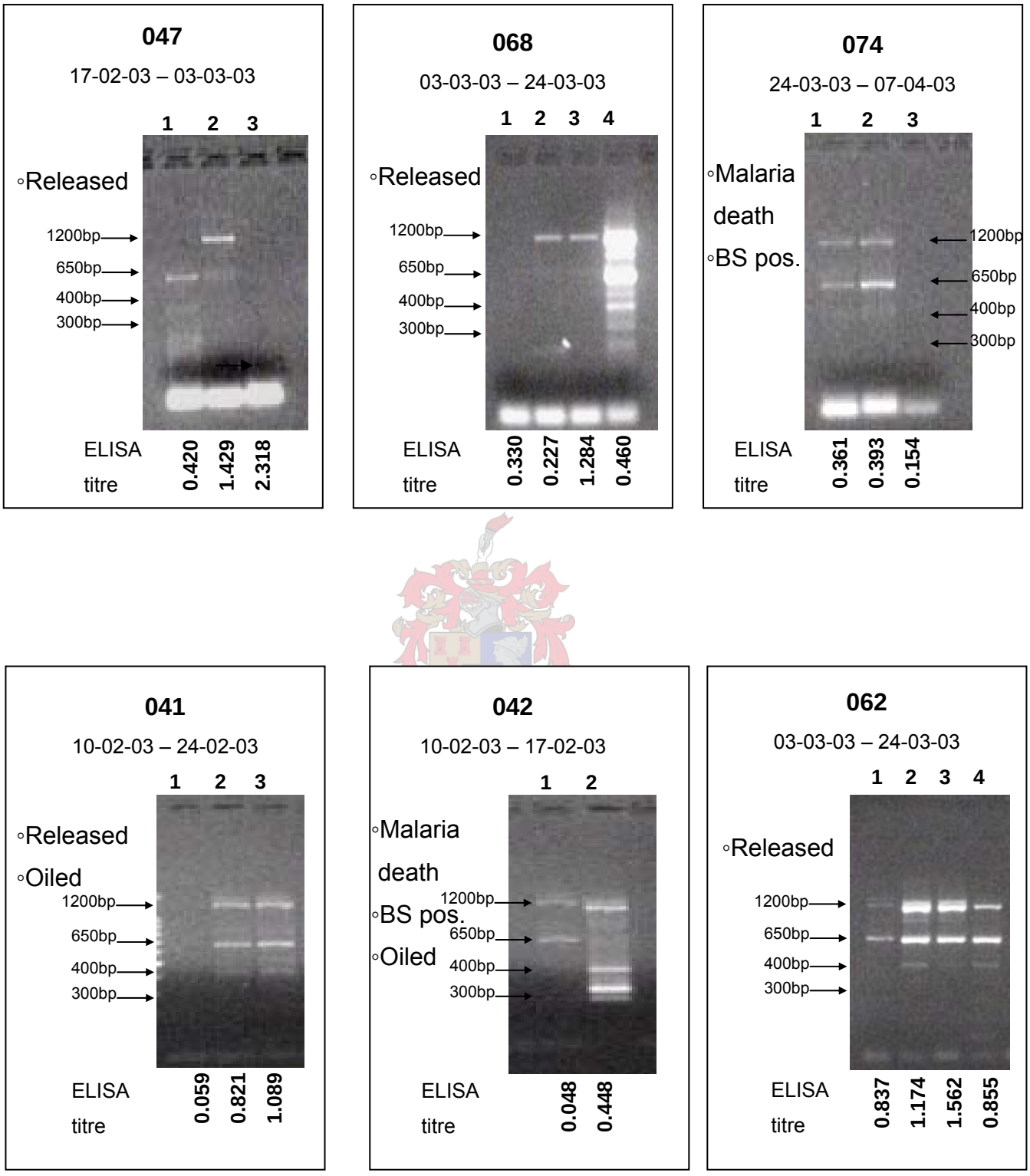


Figure 5.6: The PCR results of penguins no. 047, 068, 074, 041, 042 and 062. The date and the duration of the penguins' stay at the facility are indicated underneath the penguin number. The lanes have been numbered according to the week in (or during) which the sample has been taken and the ELISA titre value is shown underneath. On the left it is shown whether the penguin was released or died. If the penguin was oiled on admission, or blood smear positive (BS pos.) at any time during its stay, it is also indicated on the left.

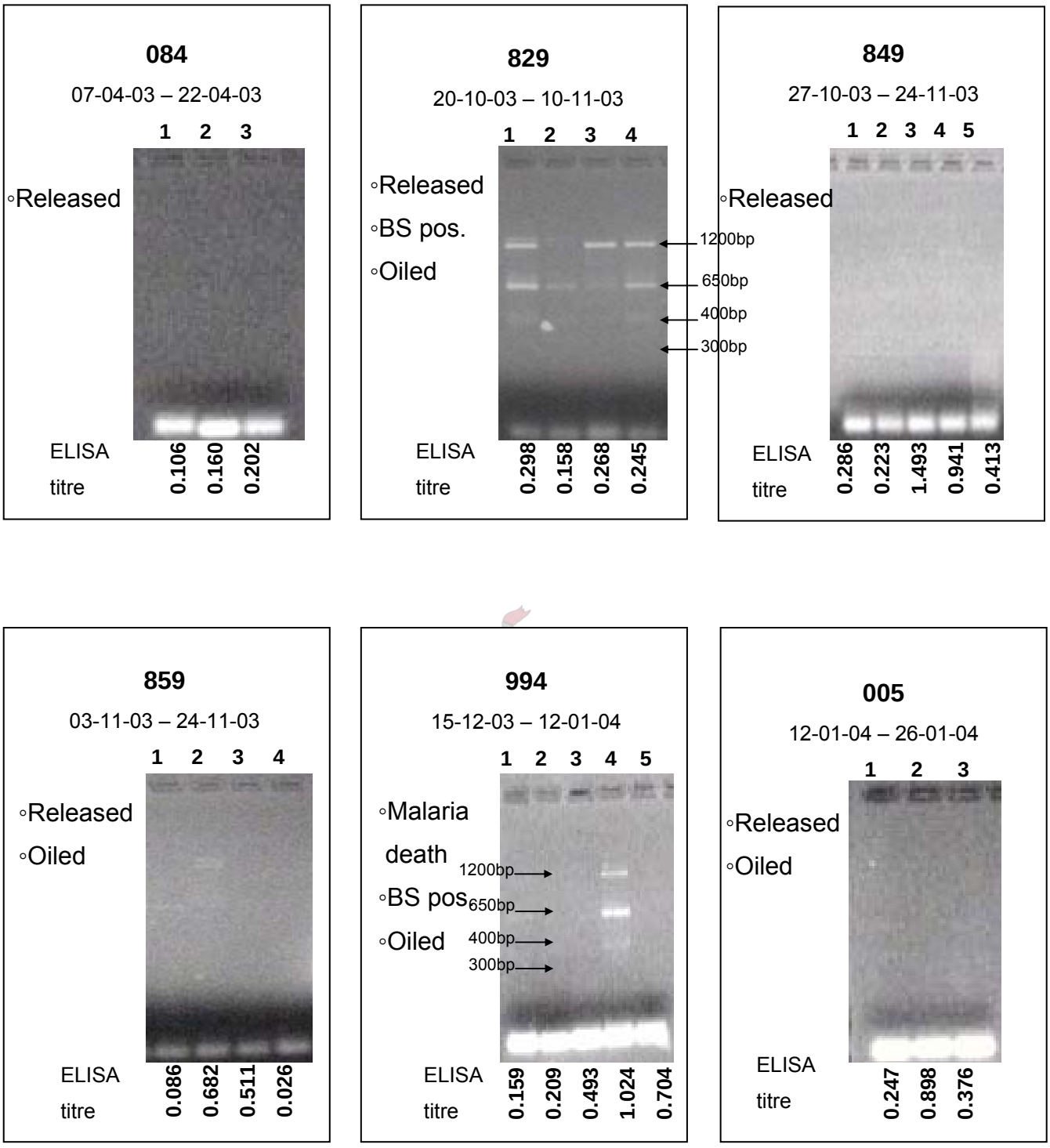


Figure 5.7: The PCR results of penguins no. 084, 829, 849, 859, 994 and 005. The date and the duration of the penguins' stay at the facility are indicated underneath the penguin number. The lanes have been numbered according to the week in (or during) which the sample has been taken and the ELISA titre value is shown underneath. On the left it is shown whether the penguin was released or died. If the penguin was oiled on admission, or blood smear positive (BS pos.) at any time during its stay, it is also indicated on the left.

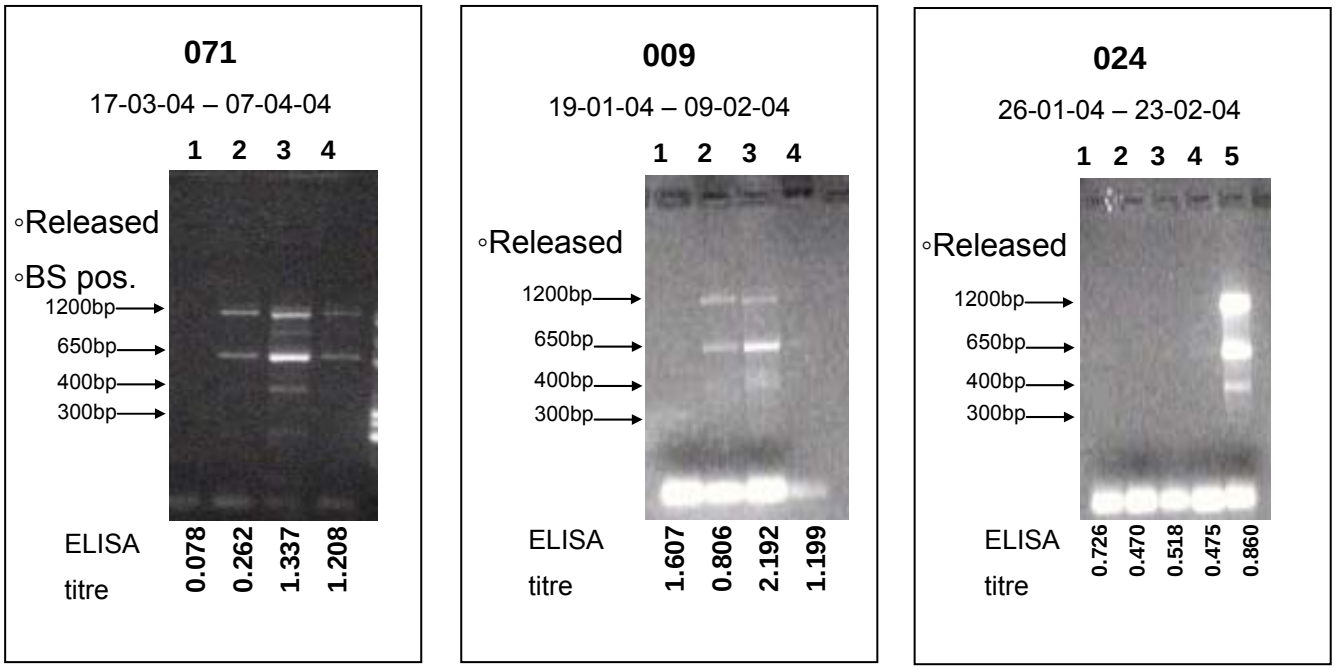


Figure 5.8: The PCR results of penguins no. 071, 009 and 024. The date and the duration of the penguins' stay at the facility are indicated underneath the penguin number. The lanes have been numbered according to the week in (or during) which the sample has been taken and the ELISA titre value is shown underneath. On the left it is shown whether the penguin was released or died. If the penguin was oiled on admission, or blood smear positive (BS pos.) at any time during its stay, it is also indicated on the left.

WINTER (MAY – OCTOBER)

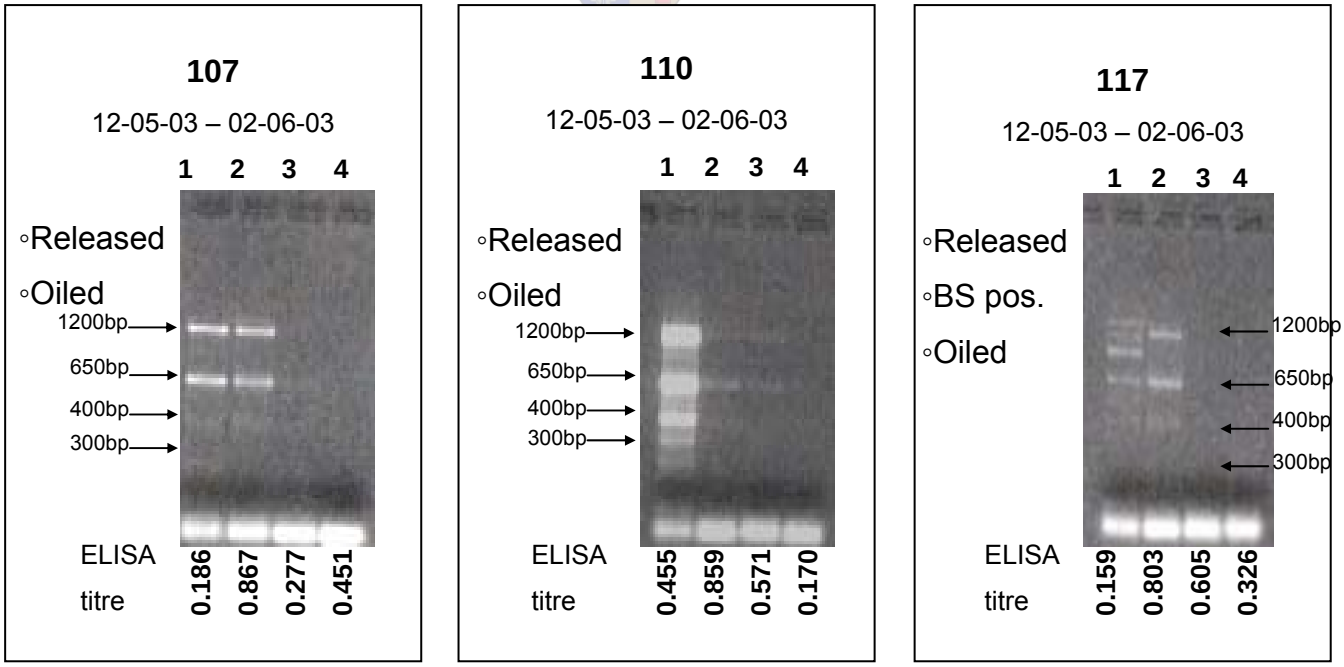


Figure 5.9: The PCR results of penguins no. 107, 110 and 117. The date and the duration of the penguins' stay at the facility are indicated underneath the penguin number. The lanes have been numbered according to the week in (or during) which the sample has been taken and the ELISA titre value is shown underneath. On the left it is shown whether the penguin was released or died. If the penguin was oiled on admission, or blood smear positive (BS pos.) at any time during its stay, it is also indicated on the left.

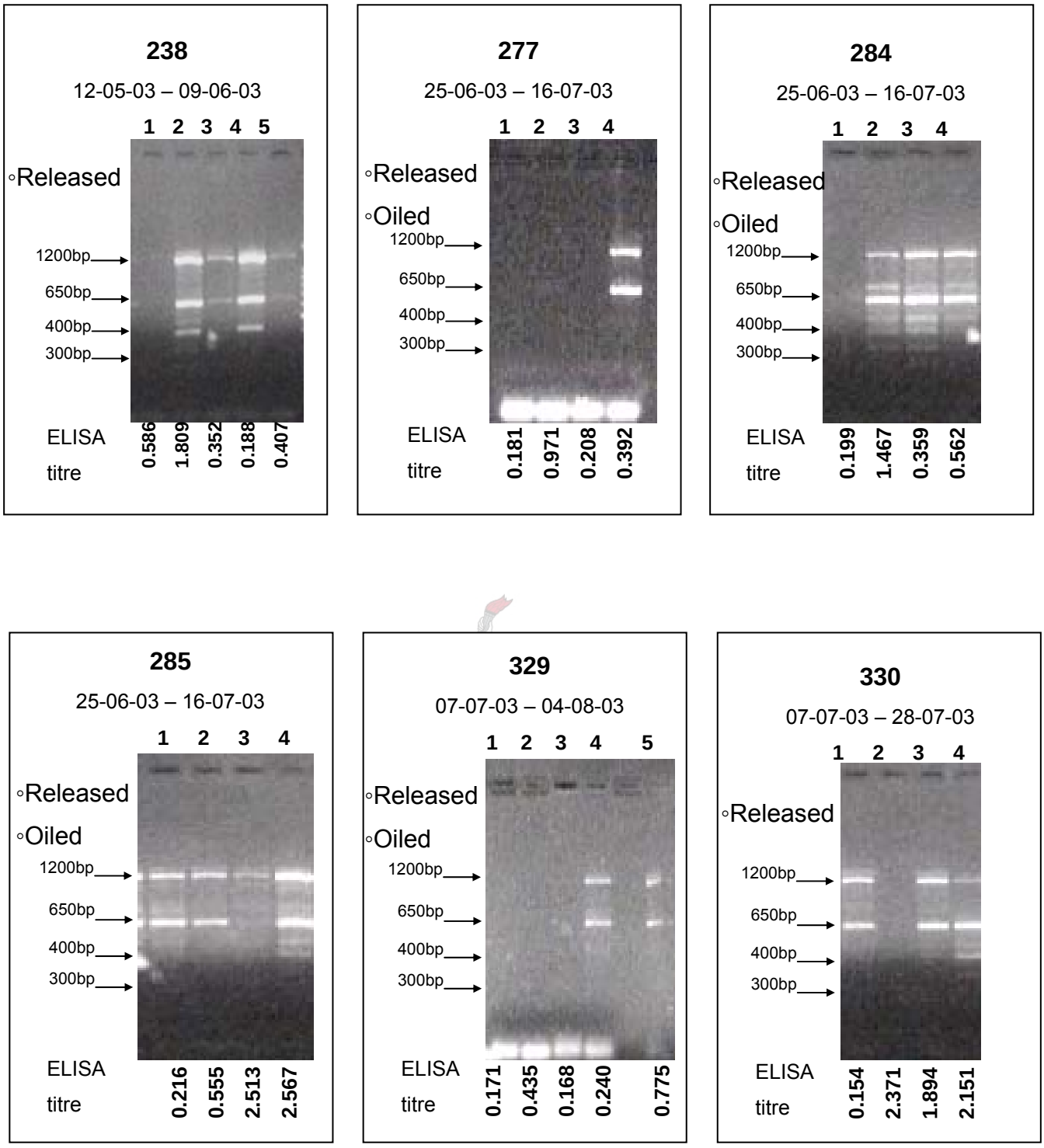


Figure 5.10: The PCR results of penguins no. 238, 277, 284, 285, 329 and 330. The date and the duration of the penguins' stay at the facility are indicated underneath the penguin number. The lanes have been numbered according to the week in (or during) which the sample has been taken and the ELISA titre value is shown underneath. On the left it is shown whether the penguin was released or died. If the penguin was oiled on admission, or blood smear positive (BS pos.) at any time during its stay, it is also indicated on the left.

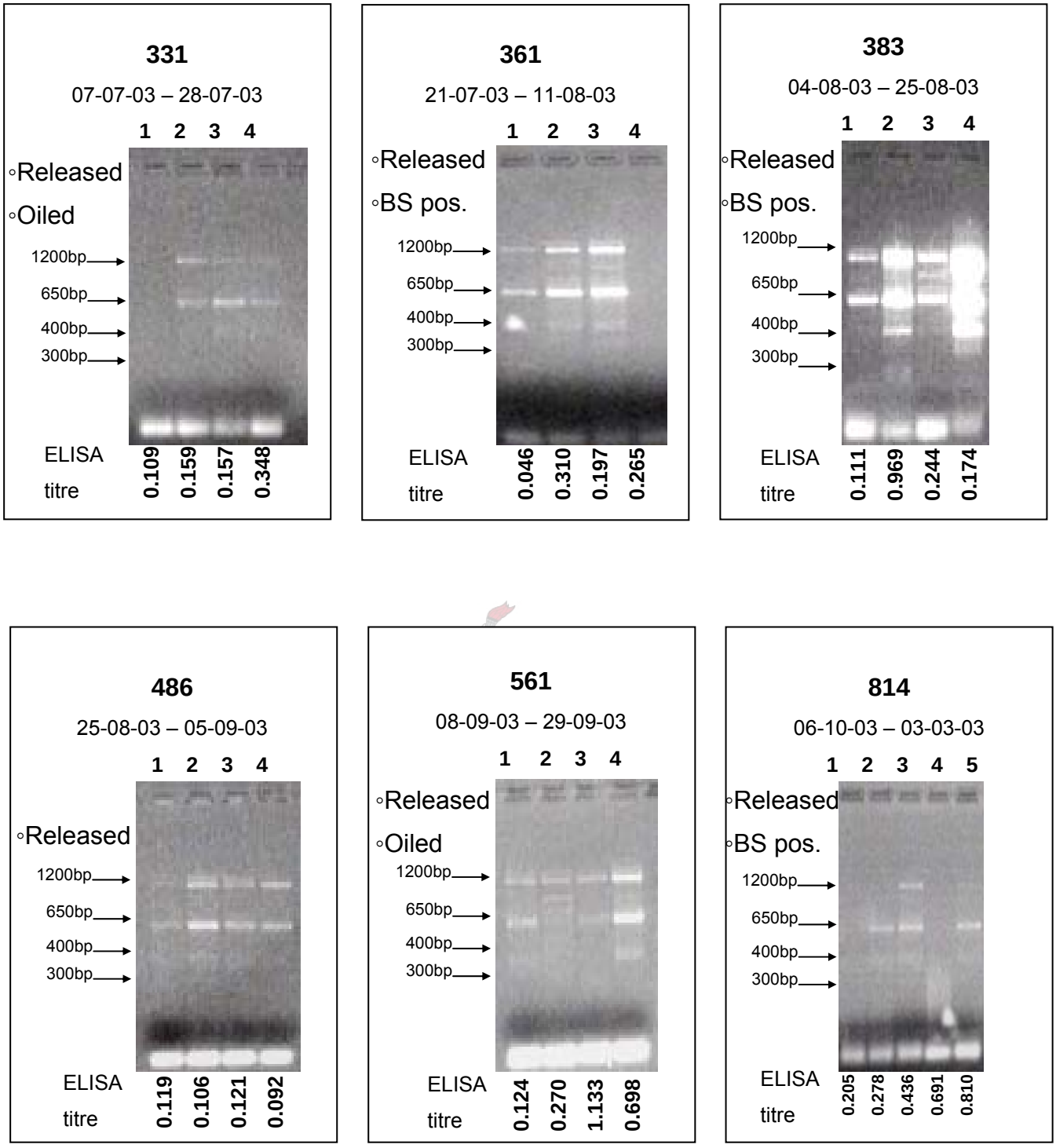


Figure 5.11: The PCR results of penguins no. 331, 361, 383, 486, 561 and 814. The date and the duration of the penguins' stay at the facility are indicated underneath the penguin number. The lanes have been numbered according to the week in (or during) which the sample has been taken and the ELISA titre value is shown underneath. On the left it is shown whether the penguin was released or died. If the penguin was oiled on admission, or blood smear positive (BS pos.) at any time during its stay, it is also indicated on the left.

The PCR was also performed on samples from five Greywing Francolins caught at Rietvlei, in order to determine if they serve as a possible reservoir for malaria, as *Plasmodium juxtanucleare* occurs naturally in francolins (Earle *et al*., 1991). The PCR results (figure 5.12) were positive for all five the francolins, confirming that they may in fact have been infected with malaria.

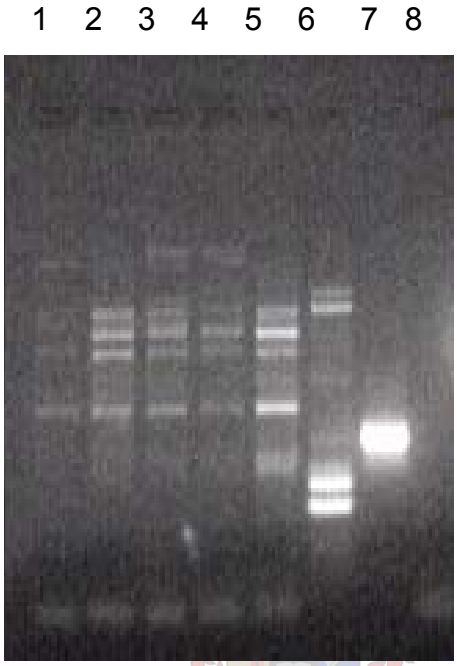


Figure 5.12: PCR amplification of Greywing Francolins in the SANCCOB area: lane 1: Francolin 1; lane 2: Francolin 2; lane 3: Francolin 3; lane 4: Francolin 4; lane 5: Francolin 5; lane 6: *Plasmodium gallinaceum*; lane 7: *Plasmodium falciparum*; lane 8: MilliQ water.

5.3. Discussion

Mortalities due to malaria were higher in the summer than in the winter. In fact, from June to September 2003 there were no malaria deaths at all. During October 2003 to January 2004 the mortalities due to malaria were higher than during February to May 2003. However, the mortality results can be viewed as biased because some of the birds that survived were given prophylactic treatment for malaria and might not have survived without the treatment. In addition, some of the birds that died did not die from malaria but from other causes.

The immunity upon admittance was relatively high throughout the year (Feb 2003 – Jan 2004) compared to the previous years (Oct 2001 – Jan 2003, Botes 2004). The immunity upon admittance only dropped slightly during August-September 2003, but

was relatively higher than that determined by Botes (2004). This phenomenon might be due to the fact that different microtiter plates were used in the two studies. The Medisorp plates (Nunc) had a slightly higher background value than the Polysorp plates (Nunc) used by Botes (2004). However, in view of the fact that background values were automatically deducted by the micro-titer plate reader, the values obtained with the Nunc Medisorp plates were therefore comparable to those obtained with the Nunc Polysorp plates. The difference in immunity upon entry is therefore most likely caused by seasonal fluctuations. During August-September there were no mortalities due to malaria and the infection rates were also low. Immunity upon admittance into the facility did not have a significant influence on the subsequent survival of penguins during rehabilitation.

The survival of penguins infected with malaria is influenced by their ability to produce an anti-*Plasmodium* immune response. The speed at which a penguin can increase the levels of serum antibodies appears to be critical for its survival. However, an increase in antibody level does not necessarily mean that the bird will survive; it must do so fast enough after infection in order to survive. In this study, the penguins that survived (and were released) and the penguins that died both showed an increase in antibody levels, indicating that both produced an immune response. The difference in immune response between these two groups was also not statistically significant, so survival did not depend on immune response alone. If birds where tested positive for malaria by blood smear, however, they were treated prophylactically for malaria. As a result this must have biased these data considerably. Had prophylactic treatment not been given, the difference in immunity response between birds that died vs birds that survived may have been significant. The reason for the mortalities during rehabilitation could rather be ascribed to the weakness of the birds on entry into the facility.

There are two possible ways in which penguins can become infected with malaria after admittance to the SANCCOB rehabilitation centre. Firstly, they may contract the disease on entry to the facility via mosquitoes. Secondly, birds may harbour malaria parasites and may suffer parasite recrudescence if weakend due to injury, oil, or stress. It is assumed that once a penguin becomes infected with malaria, it remains infected for life. If a penguin is infected with malaria and subsequently recovers, it builds up immunity to the parasite, while still continuing to carry the parasite in its tissue for life. The immune system appears to be capable of reducing the parasite to subpatent levels.

This results in a low-grade infection due to the survival of the latent parasites and is referred to as premunition. Stress factors can induce recrudescence of parasites in birds with premunition and this may be the case in some birds on entry to the SANCCOB facility.

It is assumed that birds harbouring malaria parasites enter the facility at all times of the year (not suffering from malaria infection per se). If recrudescence was therefore the primary cause of the antibody responses seen in the penguins, then the same increase in antibody level after entry would be seen throughout the year, including winter. This, however, was not the case; antibody levels during the warmer spring and summer time were high and declined during autumn, staying low during the winter. Parasite recrudescence therefore appears to play a minor role in causing infections throughout the year. The incidence of infections in winter is also very low due to infection being dependent on the presence of a suitable mosquito vector. Cooler temperatures influence the distribution of mosquito vectors negatively and subsequently also decrease malaria infections. Increases in immunity at the facility in the summer season could therefore be ascribed to infections due to the incidence of infected mosquitoes at the SANCCOB facility rather than recrudescence.

The effect of oiling on immunity was also assessed in this study. Oiling is a major threat to the conservation of the African Penguin. If an African Penguin becomes oiled it loses its insulation and cannot survive in cold water. Alternatively, if it remains on land too long it is at risk of dehydration and starvation. When ingested, oil can cause organ damage, anaemia and depression of the immune function. Depression of the immune function can possibly cause the penguins to be more susceptible to malaria infection during rehabilitation. In this study, however, the immune response of the oiled penguins compared to that of the non-oiled penguins was not found to be statistically significant. Therefore oiling was found not to influence the ability of penguins to produce an immune response. This phenomenon can probably be explained by the fact that penguins admitted to the facility because of oiling, are usually stronger than penguins admitted for other reasons (Parsons and Underhill, 2004). Penguins become oiled while spending time at sea and only strong and healthy birds will go out to fish. Non-oiled penguins include those admitted because of sickness or injury and are therefore weaker. When comparing blood smear results, there was a tendency in some of the two month periods in which the number of oiled penguins that were blood smear

positive was lower than the number of non-oiled penguins that were blood smear positive.

Penguins can also arrive at the facility already infected with malaria. Antibody level detection cannot distinguish between penguins arriving with malaria infections, and those penguins contracting the disease on entry to the SANCCOB facility. As the PCR can be used to distinguish between penguins arriving with malaria infections, and those penguins contracting the disease on entry to the SANCCOB facility, PCR assays were performed on a limited number of sera. The PCR results were separated into summer and winter results.

It was clear that the PCR assay was much more sensitive than thin blood smear tests. Thirty birds were chosen for the PCR analysis of which 9 tested positive for malaria by blood smear at any time during rehabilitation. Only four of the thirty penguins tested negative and the rest were all tested positive for malaria by PCR. Almost half (14) of the penguins arrived at the facility already infected, while twelve were either infected at the facility or suffered from recrudescence.

Three of the thirty penguins chosen for the PCR analysis died from malaria and all three died during the summer and tested positive by blood smear. Two of these birds (074 and 042) had relatively low immune responses and were already infected on arrival at the facility. A possible explanation is that the immune responses were not strong enough for these birds to survive the infection. The third penguin (994) that died due to malaria produced an immune response, although not until the fourth week. The penguin was either infected at the facility or suffered from recrudescence and did not produce an immune response fast enough to overcome the infection.

During the summer months, five (out of 15) penguins contracted malaria at the facility or suffered from recrudescence. When comparing the PCR results with the ELISA titre values observed for each of these five birds (047, 062, 041, 071, 009) a generalised pattern emerges. The malaria infection (as detected by PCR) appears first, where after the immune response was found to increase and only after the malaria infections started to decrease or disappeared completely did the immune response start to decrease. Penguins that were infected before arrival to the facility in the summer ((074, 062, 829) had relatively high ELISA titre values on arrival. Their immune responses also increased and only started to decrease after the malaria infection decreased or

disappeared. However, penguin 829 had a relatively low immune response during the four weeks of rehabilitation even though it was infected with malaria during the whole period. This may be due to higher T-cell immunity which was not measured by the ELISA. Its survival may also have been brought about by the prophylactic treatment it received.

During the winter months, 9 (out of 15) penguins arrived at the facility with malaria infections. From these results it can be deduced that malaria infections do in fact occur in breeding colonies in winter. The reason for such an apparent high prevalence during the winter time was probably due to the fact that penguins with high titre values were selected for the PCR analysis. The other six penguins contracted the infection at the facility. This was probably due to recrudescence and not because of infection via mosquitoes due to the fact that it was winter during which mosquitoes are not normally present. Similar to penguin 829, penguins 331, 361 and 486 had very low immune responses compared to the other infected penguins. All four of these penguins survived the rehabilitation and were released. A possible explanation might be that they have previously been infected with the same type of malaria and that they were capable of reducing the infection to subpatent levels or that the prophylactic treatment (in birds 829, 361) lead to their survival.

An interesting phenomenon can be seen in penguins number 829, 238, 285, 330 and 814. All five of the penguins have positive PCR results for one or more weeks, after which the PCR band disappears or fade. In the week thereafter, though, the PCR bands reapprear. A possible, although unlikely explanation might be, that the DNA extraction procedure was not successful for those samples. Another explanation may be that the penguins received anti-malarial treatment before the blood samples were taken and that the number of parasites in the blood was decreased. However, before the next sample was taken, the number of parasites increased again, producing a positive PCR result.

It is assumed that when penguins are kept in restricted areas, endemic malaria can be transmitted to them (Brossy 1992). It is doubtful that malaria in penguins would occur in absence of infection in wild birds, since penguin infection occurs during periods of seasonally high infection rates in wild birds. Nevertheless, penguins cannot be ruled out as potential reservoirs. The PCR assays performed on the Greywing Francolins

indicated that all of the five francolins tested were infected with malaria. It is therefore possible that the Greywing Francolins occurring in close proximity to the facility can act as an avian malaria reservoir.

The results obtained in this study, indicate that most penguins admitted to the SANCCOB facility in summer usually become infected via mosquitoes. In the apparent absence of mosquito vectors in winter, the infection rates in winter appear almost negligible and infections in the facility may rather be ascribed to parasite recrudescence. Conversely this means that recrudescence appears to play a minor role in malaria infection during rehabilitation. It is also clear that penguins can arrive at SANCCOB infected with malaria. This indicates that they can also become infected with malaria in the breeding colonies. Consequently, an investigation into the prevalence of avian malaria in the breeding colonies was launched and the results of the investigation will be presented in the next chapter.

CHAPTER 6

INVESTIGATIONS INTO AVIAN MALARIA IN THE AFRICAN PENGUIN IN BREEDING COLONIES

6.1. Introduction

The African Penguin occurs along the southeastern and southwestern shores of South Africa and Namibia. Its breeding range extends from Hollamsbird Island, off central Namibia, to Bird Island in Algoa Bay, even though non-breeding birds often disperse as far as KwaZulu-Natal and southern Angola. African Penguins currently breed at 27 colonies, including 26 islands colonies and three mainland sites.

The prevalence of parasitemias of wild African Penguins at the southern coast of South Africa was 7%, 5% (Fantham & Porter, 1944), and 0.7% (Brossy, 1992), as determined by studies based on the examination of blood smears. A study by Graczyk *et al.* (1995) based on an enzyme-linked immunosorbent assay (ELISA), detected a 52% seroprevalence of malarial antibodies in African Penguins from the Boulders, Simon's Town colony. Another study by Graczyk *et al.* (1995) included breeding colonies at Robben Island, Dassen Island and Boulders, Simon's Town, and detected a seroprevalence ranging between 29% and 35%. An accurate assessment of naturally occurring exposure to avian malaria in wild African Penguins is important in understanding the role that the disease plays in the population. It is also crucial to evaluate the risk of releasing possibly infected rehabilitated penguins into the wild.

The aim of this study was to investigate avian malaria in African Penguins in breeding colonies by means of antibody detection and PCR analysis. In order to obtain a suitable representation of breeding colonies, two island colonies and one mainland colony were chosen for this study namely Robben Island, Stony Point (Bettys Bay) and Dassen Island.

Robben Island, with a surface area of 507 ha, is the largest island along the coastline of South Africa. It is roughly oval shaped and about 2 km from north to south. The island is fairly flat with the highest point on the island, Minto Hill, only about 30 m above sea

level. The island has been exploited for the last three and a half centuries and has been used for many purposes including seals and seabird exploitation, agriculture, a military base, a place for isolation of lepers and as a notorious prison. For historical reasons, the whole island is now a museum, the Robben Island Museum, and also a World Heritage Site.

The original colony of African Penguins on Robben Island was exterminated by 1800 and penguins only started to recolonize the island in 1983. Robben Island is regularly used as a release site for penguins that have been rehabilitated at SANCCOB. Few of these have remained to breed, but their presence may have stimulated young birds to take up residence and start a colony. The Robben Island penguin population has grown to 15 000 and with 5 700 breeding pairs of penguins on the island, it is the third largest colony of African Penguins (Hockey 2001, Underhill 2004a).

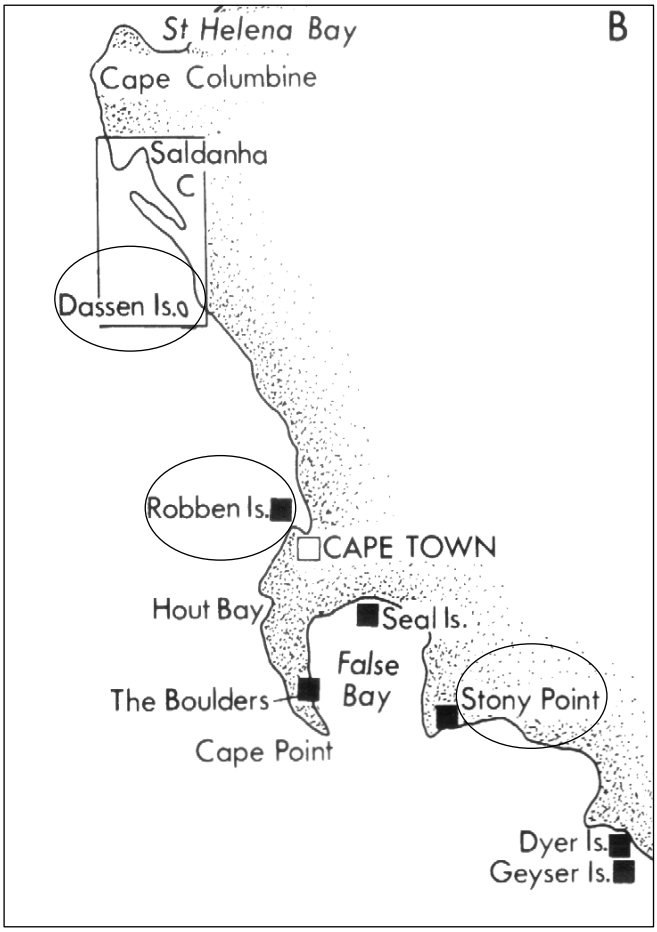


Figure 6.1: Map of the breeding colonies of the Afican penguin close to Cape Town. Circles indicate the breeding colonies chosen for this study (Randall 1989).

Stony Point lies within the village of Betty's Bay, about 90 km from Cape Town, just east of Hangklip. This penguin colony only started in 1982 and by 1986 there were about 40 nests. At present the colony consists of about 150 pairs. As determined by flipper banding the most penguins came from Dyer Island, 60 km farther east. It is unlikely for penguins to switch colonies once they start breeding; consequently the colonists are almost certainly young penguins from Dyer Island that established themselves at Stony Point. Stony Point was fenced off in 1987 in order to reduce disturbance from people and predators (Hockey 2001, Underhill 2004b).

Dassen Island is the second largest offshore island at 273 ha. It lies 9 km off the coast opposite the village of Yzerfontein, 55 km from Cape Town. The island is generally flat and sandy with patches of exposed bedrock and huge granite boulders strewn in some areas. Dassen Island is well known for the decline in the penguin population due to egg collection and guano exploitation. At the start of the 20th century, Dassen Island was home to well over a million African Penguins, however, in May 2000 only 15 000 penguins were counted on the island. At the moment this penguin population is the largest colony of African Penguins. Dassen Island is a reserve of the Western Cape Nature Conservation Board (Hockey 2001, Underhill 2004c).

6.2. Materials and methods

6.2.1. Sample collection

Samples were collected from three selected breeding colonies at: Robben Island on 11-03-04, Dassen Island on 15-03-04 and Stony Point at Betty's Bay on 12-03-04 (See figure 6.1). Sample collection took place at the time of year when high malaria infection rates were observed at SANCCOB. A total of 51 samples were collected from each breeding colony using filter paper strips and prepared as described in section 5.2.1. Samples were also collected from Bird Island, Algoa Bay in the Eastern Cape Province by Albert Schultz. A total of 12 samples were collected during September 2004 also using filter paper strips and prepared as described in section 5.2.1.

6.2.2. ELISA for penguin anti-*Plasmodium* antibodies

The ELISAs were performed as described in sections 5.2.2 to 5.2.5.

6.2.3. PCR analysis

The PCR assay was performed on all the samples collected from the breeding colonies. The DNA was extracted as described in section 5.2.7.1 and the PCR was performed as described in section 2.5.7.2.

6.3. Results

Penguin blood samples were collected at the three breeding colonies. At Robben Island 51 penguins were bled in total. They were all adult birds and mostly non-breeders. At Stony Point 51 penguins were bled. Five of these were juveniles and the rest were adults (46). Lastly, 52 penguins were bled at Dassen Island; 18 juveniles, 4 chicks and the rest were all adults (30). All the samples collected from the breeding colonies were evaluated for malaria by thin blood smear and the prevalence of malaria were found to be 11%, 9% and 34% for Robben Island, Stony Point and Dassen Island respectively.

The ELISA results were separated into adult birds, juveniles and chicks for each respective breeding colony so as to determine whether maternally transmitted antibodies could contribute to the antibody levels of these birds (figure 6.2).

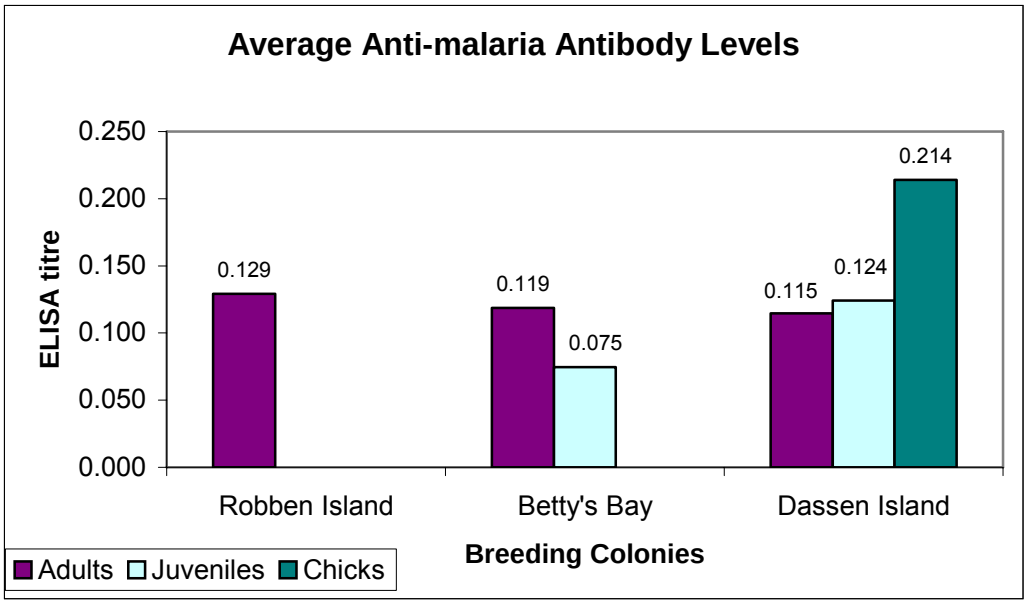


Figure 6.2: The average anti-malaria antibody levels of the penguins in breeding colonies at Robben Island, Stony Point and Dassen Island. Data were separated into adults, juveniles and chicks.

The difference in the average anti-malaria antibody levels of the different breeding colonies was not found to be statistically significant. At Dassen Island, which was the only colony in which blood samples were collected from chicks, the difference in the average immune response of chicks and the juvenile birds was found to be statistically significant (P = 0.0251). This indicated that maternally transmitted antibodies could contribute to the antibody levels in the serum of penguin chicks. The ELISA titre values were on average lower than those from penguins at SANCCOB (Chapter 5). The seroprevalence of malarial antibodies in African Penguins were 13%, 9% and 11% at Robben Island, Stony Point and Dassen Island respectively using a cut-off absorbance level of 0.20 as positive (this cut-off value was also used in the interpretation of SANCCOB results).

The PCR analysis was performed on all the samples collected from the breeding colonies. The results can be seen in figures 6.3, 6.4 and 6.5. The ELISA titre values obtained for the different penguins are indicated below the PCR result. If the penguin tested positive for malaria by thin blood smear it is indicated with a "•". The prevalence of malaria as determind by PCR analysis was 88%, 94% and 90% for Robben Island, Stony Point and Dassen Island respectively. The data obtained in this study is summarised in Table 6.1.

Table 6.1: A summary of data obtained from the breeding colonies at Robben Island, Stony Point and Dassen Island.

Number of penguins	**Robben Island 11/03/04**	**Stony Point 12/03/04**	**Dassen Island 15/03/04**
Total	51	51	52
Adults	51	46	29
Juveniles/ Blues/ Chicks	0	5	23
Blood smear positive	6 (11%)	5 (9%)	18 (34%)
Average ELISA titre	0.129	0.114	0.126
ELISA positive	7 (13%)	5 (9%)	6 (11%)
PCR positive	46 (88%)	48 (94%)	47 (90%)

Robben Island (11/03/04)

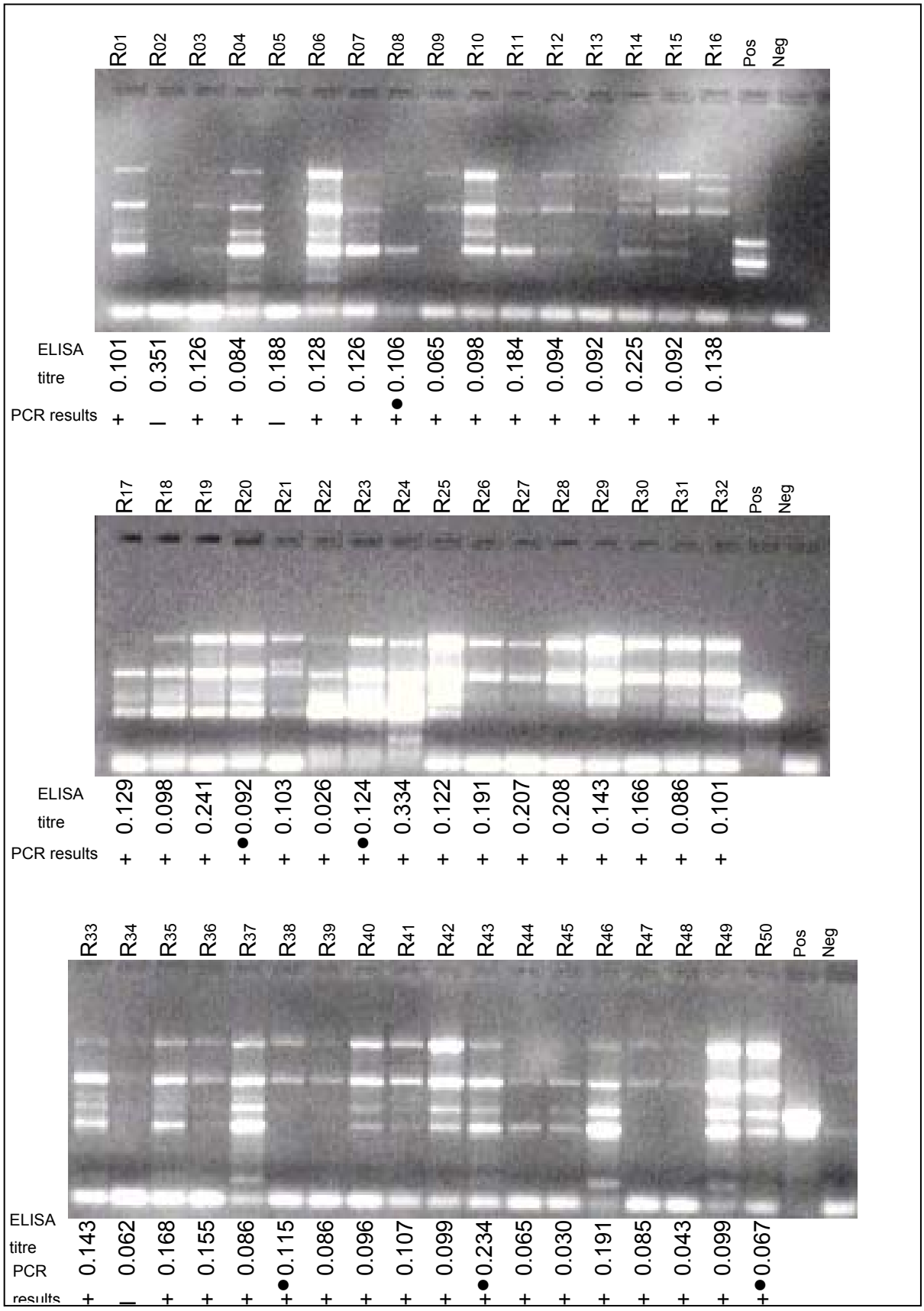


Figure 5.3: PCR amplification of samples collected from Robben Island. Electrophoresis of the amplified fragments on a 2% agarose gel is shown. ELISA titre values are indicated underneath. Penguins that tested positive by thin blood smear are indicated with ●.

Stony Point, Betty's Bay (12/03/04)

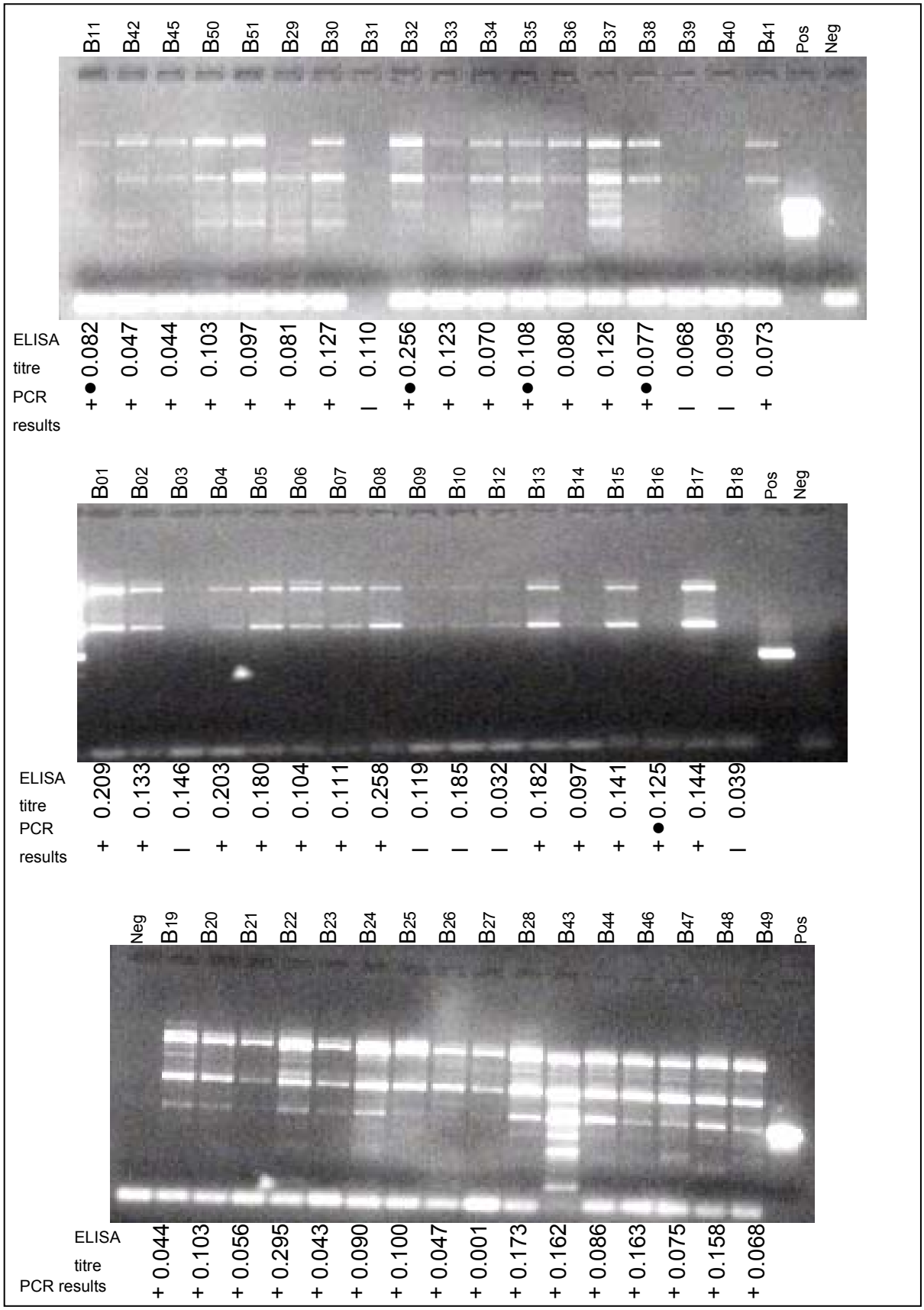


Figure 5.4: PCR amplification of samples collected from Stony Point. Electrophoresis of the amplified fragments on a 2% agarose gel is shown. ELISA titre values are indicated underneath. Penguins that tested positive by thin blood smear are indicated with •.

Dassen Island (15/03/04)

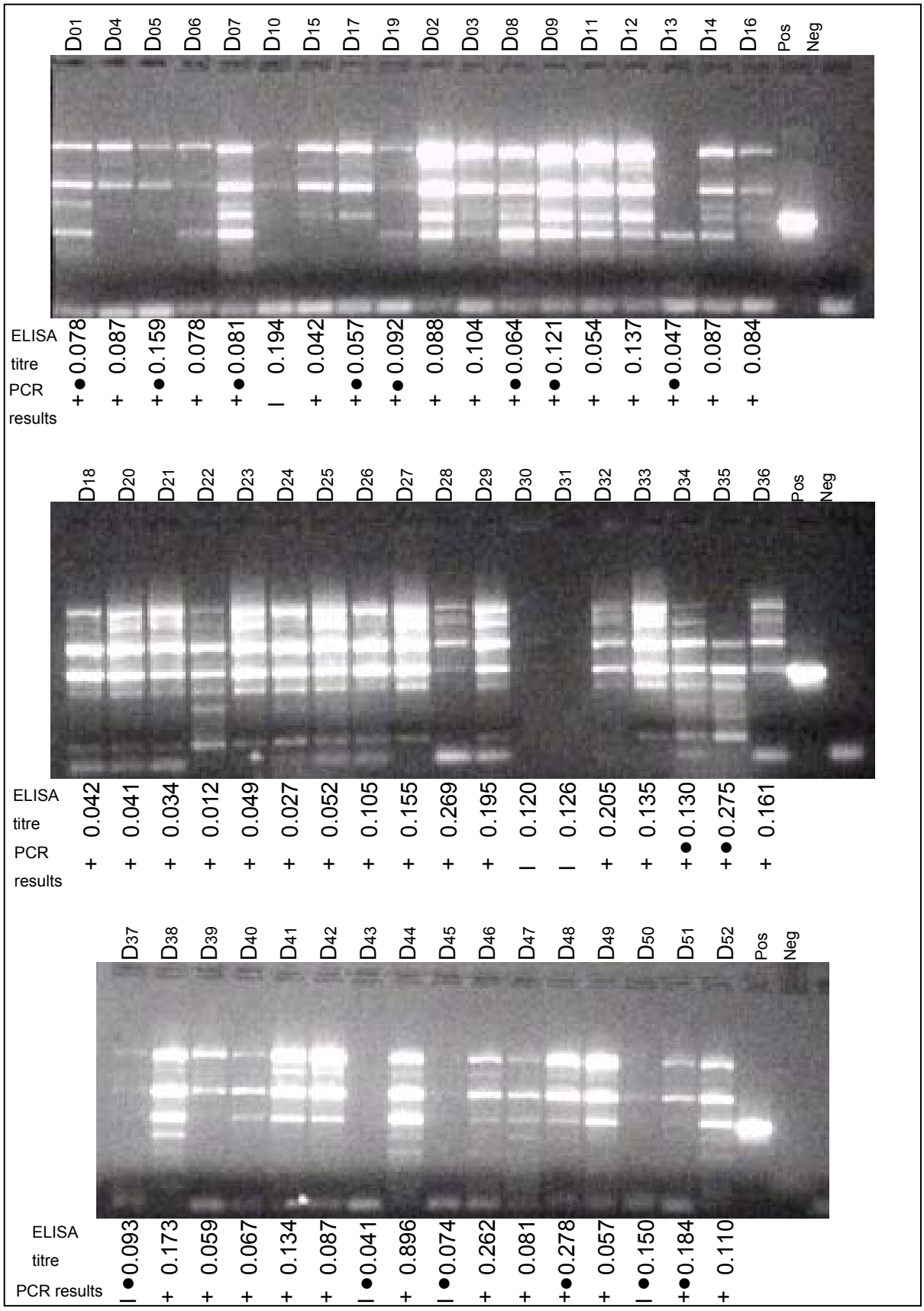


Figure 5.5: PCR amplification of samples collected from Dassen Island. Electrophoresis of the amplified fragments on a 2% agarose gel is shown. ELISA titre values are indicated underneath. Penguins that tested positive by thin blood smear are indicated with •.

The samples received from Bird Island were also analysed using ELISA and PCR. The results can be seen in figure 6.3. The seroprevalence of malarial antibodies in African Penguins at Bird Island was 83% and the prevalence as determined by PCR was 91%.

Bird Island, Algoa Bay

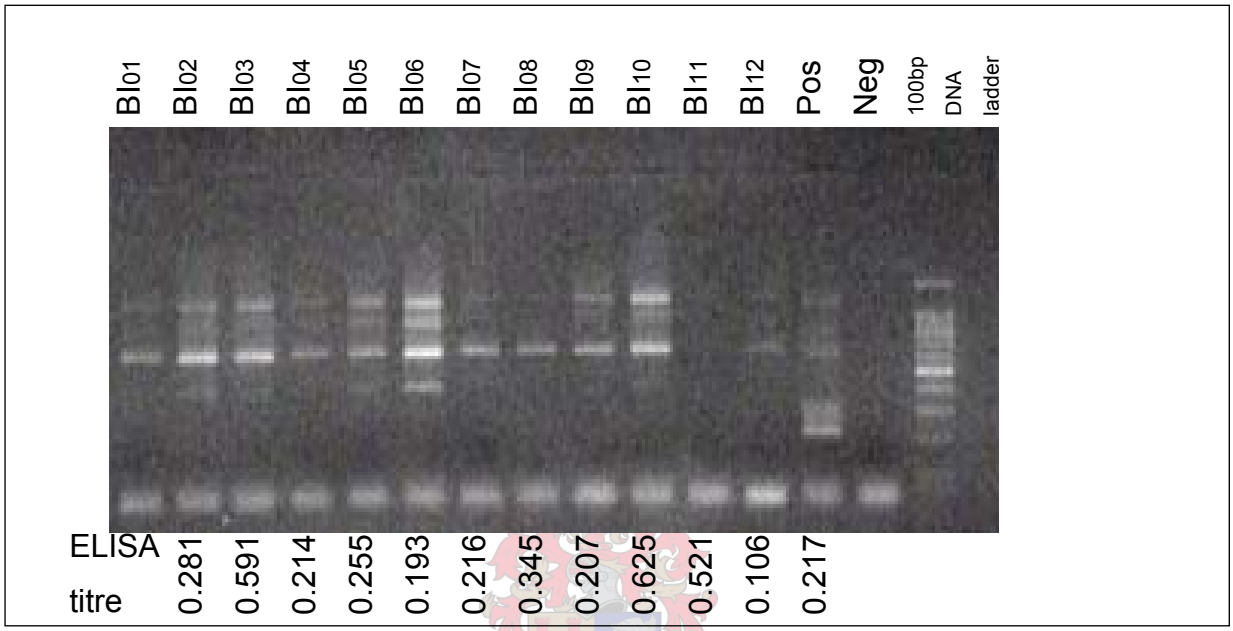


Figure 6.3: PCR amplification of blood samples collected from Bird Island. Electrophoresis of the amplified fragments on a 2% agarose gel is shown. ELISA titre values are indicated underneath.

6.4. Discussion

The global distribution of warm water penguins overlaps with the occurrence of *Culex* species mosquitoes (Stonehouse 1975, Knight and Stone 1977). Contact with vectors is less likely for coldwater penguins whose distribution is only partially covered by the occurrence of *Culex* species. Most of the species of wild penguins breed in areas free of malaria vectors and these penguins are naïve to species of *Plasmodium* (Bennett *et al.*, 1993). Penguins in areas with no (documented) *Culex* spp. occurrence may become exposed while in other locations or during migration (Graczyk *et al.*, 1995). Rescued wild penguins may contract malaria while being rehabilitated at SANCCOB where after they return to their own colonies when released (Brossy 1992). Over 87% of rehabilitated African Penguins return to their former nests and mates (Randall 1980). Seropositivity of wild penguins may be generated by a single contact with the malaria parasite because of persistence of subclinical infection in penguins (Cranfield *et al.*,

1994). Seropositivity may also be due to maternally transmitted antibodies in penguins younger than two months (Graczyk *et al.*, 1994a). For these reasons, malarial seropositivity in penguins from breeding colonies may not fully reflect the exposure to parasites in their natural habitat.

Although the antibody levels of only a few chicks were analysed in this study, these showed elevated levels compared to juveniles from the same colony (Dassen Island), providing evidence that these antibodies against malaria were maternally transferred and not the result of malaria infection. However, the elevated antibody levels in adult and juvenile penguins in all the breeding colonies indicated that many of these birds were indeed infected with malaria. The seroprevalence of the malaria antibody levels correlated reasonably well with the prevalence as determined by blood smear results. In general, mean ELISA titre values of penguins from the breeding colonies were lower than those of captive penguins at the SANCCOB facility.

The PCR results showed a very high prevalence (88% - 94%) of malaria in the breeding colonies at Robben Island, Stony Point and Dassen Island. This indicated that PCR assays are much more sensitive than both thin blood smears and antibody level detection for malaria diagnosis. The PCR results and the antibody levels do not seem to correlate. This might, however, be explained by the generalised pattern of infection deduced by means of comparing PCR results (infection rate) with ELISA titre values at SANCCOB as described in the previous chapter. At SANCCOB it was possible to follow the antibody level and infection rates of individual penguins for a period of a few weeks. A generalised pattern of infection rate and antibody level emerged; the malaria infection appears first, where after the immune response increased and only after the malaria infection decreased or disappeared completely did the antibody levels decrease as well. At the breeding colonies it was only possible to obtain a single determination for each bird. Penguins at the breeding colonies with a high infection rate and a low antibody level may have been newly infected and the sample may have been taken before the antibody levels started to increase. In contrast, penguins with a high antibody level and low infection rate, may have been infected and overcome the infection by reducing the number of parasites, but the sample was taken before the antibody level decreased and therefore still shows a high antibody level.

Penguin blood samples from Bird Island indicated that malaria also occurs at this breeding colony. Bird Island is a group of 4 islands in Algoa Bay. In the 1930s less than 100 pairs of African Penguins were breeding on the Island, having been decimated by egg collection and other human disturbances. However, since the cessation of guano scraping, penguins thrive on Bird Island, and the population presently consists of over 10 000 birds. The seroprevalence of malarial antibodies in African Penguins at Bird Island was 83% and the prevalence as determined by PCR was 91%. Unfortunately there were no blood smear results for these samples. Interestingly, these samples were taken during September and therefore during springtime when malaria was not expected. A possible explanation might be that because Algoa Bay is situated on the Eastern coastline it is relatively warm during the winter and spring during which fresh water (essential for mosquito proliferation) may be available.

The antibody levels as well as the PCR results indicate that an alarmingly high number of penguins were infected with malaria in the breeding colonies at the time at which they were tested (late summer). In fact, almost all the birds tested positive for malaria by PCR. The timing of sample collection was specifically chosen during a period when high malaria infection rates did occur at SANCCOB, and could be expected to be lower during other periods of the year. It was always thought that malaria infection in wild penguins was limited by the presence of suitable arthropod vectors (mosquitoes). Breeding colonies do not appear to favour the proliferation of mosquitoes. The islands have no fresh water essential for breeding, except during the rainy season when pools of fresh water are known to lie stagnant for quite long periods, while the prevalence of onshore south-eastern winds in the summer at Stony Point may hamper the mosquito's ability to distribute itself at this mainland breeding colony. However, according to this study, the malaria prevalence in the breeding colonies was between 88% and 94%. This raises the question as to how these penguins became infected. As mentioned before, wild penguins may become infected with malaria while being rehabilitated at SANCCOB and can possibly be exposed while in other locations during migration. It is, however, doubtful that these two explanations are solely responsible for the high malaria prevalence observed in the breeding colonies. Another possibility might be that malaria is distributed via a different vector in the breeding colonies; for instance, on Dassen Island many of the penguins carried fleas. Is it possible that malaria can be distributed via fleas on Dassen Island, even though no vector other than mosquitoes has never been reported? A study by Graczyk *et al.* (1995) included Gentoo penguins

Pygoscelis papua and King penguins *Aptenodytes patagonicus* from Kerguelen and Crozet Island. These penguins had a seroprevalence of malaria antibodies of 33% and 58% respectively, although no *Culex* spp. mosquitoes were found at Crozet and Kerguelen islands (Crafford *et al*. 1986). However, a tick species (*Ixodes uriae*) parasitizing the penguins has been reported (Chastel *et al.* 1993). Nevertheless, further research is essential before any deductions can be made from the results obtained in this study.

As a final point, no mortalities or sick birds were observed at the breeding colonies even though it was clear that there was a high malaria prevalence. This raises the question of whether a different malaria species occurs at SANCCOB than in the breeding colonies. It is possible that *P. relictum* and *P. elongatum* are responsible for malaria at the breeding colonies and that the penguins have evolved with these parasites and are capable of combating and controlling such infections. At SANCCOB, penguins may be exposed to the mainland avian malaria species, *P. juxtanucleare,* which their immune systems have not adapted to, and therefore may be the reason for the mortalities occurring there.

If penguins are exposed to different, less virulent malaria species in the breeding colonies, it may also explain why the average antibody level is lower in penguins from breeding colonies than captive penguins at SANCCOB. As mentioned before, a common feature of *P. relictum* and *P. elongatum* in African Penguins, is that the virulence of *P. relictum* has always been higher than that of *P. elongatum* (Graczyk *et al*., 1994), indicating that different *Plasmodium* species can have a different epidemiology as well as a different degree of disease severity. Island colonies may therefore have very different malaria epidemiologies to those on the mainland where they may additionally be exposed to *P. juxtanucleare*.

Another fact that must be kept in mind is that penguins admitted to the facility are usually oiled, injured, sick or weak. Even if these penguins are capable of coping with infections in the wild when they are healthy, oiling or sickness can break down their immunity and they can succumb to the malaria infection.

In conclusion, it is clear that malaria occurs within the breeding colonies. The lack of sick and weak penguins in these colonies may indicate that the penguins are infected with different *Plasmodium* species than at SANCCOB, and it may even be possible that

a different vector is responsible for the distribution of malaria on in the breeding colonies.

CHAPTER 7

CONCLUSION AND FUTURE PERSPECTIVES

The malaria infection rate of penguins at the SANCCOB rehabilitation facility during the warmer spring and summer time was higher than during the colder autumn and winter months. This coincided with the anti-*Plasmodium* immune responses that demonstrated a higher increase after admittance during spring/summer than during autumn/winter. The increase in immunity at the facility during the summer months can largely be ascribed to infections due to the incidence of infected mosquitoes at the SANCCOB facility. If recrudescence was the primary cause of malaria infections, it would be expected that recrudescence should occur at any time of the year. The incidence of infections in the winter was, however, very low, and it appears that recrudescence therefore plays a minor role.

The ability of penguins to combat *Plasmodium* infections is influenced by their ability to produce an anti-*Plasmodium* immune response. However, in this study, there was no statistically significant difference between the immune responses of penguins that died and those that survived, indicating that antibody levels did not play a significant role in survival at the facility. The effect of oiling on immunity was also assessed in this study and was found not to influence the ability of penguins to produce an immune response. However, penguins admitted to the facility because of oiling are usually stronger than penguins admitted because of sickness or injury. This data may also have been biased due to the prophylactic treatment of malaria positive penguins.

There are three ways in which penguins can become infected with malaria at the SANCCOB facility. Penguins can become infected at the facility via mosquitoes or suffer from parasite recrudescence. It is also possible that the penguins can arrive at the facility previously infected with malaria. PCR analysis showed that almost half of the penguins tested were infected with malaria on arrival to SANCCOB, indicating that avian malaria may be present in the breeding colonies.

Investigations into avian malaria at the breeding colonies were performed at a time of year when the highest frequency of infections occurred at SANCCOB. These investigations at Robben Island, Stony Point and Dassen Island revealed that avian

malaria definitely occurs in wild penguin colonies. The avian malaria prevalence was found to be extremely high (88%- 94%), even though there were no sick birds or mortalities observed at the breeding colonies. This raises the question of why there are only mortalities at SANCCOB, if malaria occurs in both the breeding colonies and the SANCCOB facility.

Penguins can be infected with three types of malaria i.e. *P. relictum, P. elongatum* and *P. juxtanucleare* respectively. Each is hypothesised to have a different epidemiology and a different degree of disease severity. It is assumed that *P. relictum* and *P. elongatum* occur naturally in breeding colonies. Alternative, *P. juxtanucleare* has only recently been found to cause mortalities at the SANCCOB facility. Greywing Francolins, the natural host for *P. juxtanucleare*, were captured at Rietvlei and tested positive for malaria by blood smear and by PCR. It is possible that francolins can act as a possible avian malaria reservoir for *P. juxtanucleare*. As penguins have previously only occurred on offshore islands, it may be that penguins are exposed to this particular pathogenic avian malaria species on the mainland and that this may be the reason for mortalities at the SANCCOB facility. It is also possible that penguins can become infected with more than one *Plasmodium* species. These multiple infections are possibly indicated by the PCR results, where the multiple bands suggest that more than one *Plasmodium* species was present in the blood sample. The infection of penguins at the SANCCOB facility with *P. juxtanucleare* and the subsequent release of these penguins back into nature may result in the introduction of this land avian malaria species into the breeding colonies. This may have dire consequences for the African Penguin in the long-term as they may not have the ability to combat this pathogen to which they have not been exposed to in the past.

Future research should therefore be directed at determining which *Plasmodium* species is mainly responsible for malaria infection at the SANCCOB facility and in the breeding colonies. This work would have to include a genomic investigation to identify unique sequences in the genomes of the three malaria species, which in turn could then be used for the development of specific diagnostic PCR assays for the identification of the malaria species that is primarily responsible for the infection of penguins at the SANCCOB facility and at the breeding colonies.

This study confirms that a large proportion of penguins admitted to the SANCCOB facility in summer are infected with malaria there. This evidence thus shows that the position of the facility next to Rietvlei places the penguins at a greater risk of infection, and that the SANCCOB facility may have to be moved to overcome this problem. Further evidence in which the incidence of specifically *P. juxtanucleare* infection can be shown would, however, prove conclusively that the facility should be moved.

In conclusion, global warming may bring about a significant increase in malaria prevalence, which in turn may have an immense influence on the African Penguin population. Climate change may enable the range or density of invertebrate vectors to be increased. Additionally, the development of *Plasmodium* parasites within mosquitoes is temperature-dependant and there is a threshold temperature below which *Plasmodium* cannot develop to its infective stage. In Hawaii, the threshold temperature for transmission has been estimated to be 13°C (Benning *et al*. 2002). The predicted 2°C warming in global mean air temperature could therefore greatly increase the range of avian malaria (Patz & Reisen 2001, Kovats *et al*. 2003).

As mentioned in the previous chapter there is no fresh water in the breeding colony areas except during rainy seasons when pools of fresh water may lie stagnant for quite long periods. In view of the global warming trend, temperatures during these rainy seasons may increase to a point that it may be possible for mosquitoes to proliferate, enabling malaria to be spread from one penguin to another.

Therefore, an accurate assessment of naturally occurring exposure to avian malaria in wild African Penguins at breeding colonies is important in understanding the population dynamics of this endangered species. It is also crucial to evaluate the risk of releasing possibly infected rehabilitated penguins into the wild.

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APPENDIX

1. STATISTICAL ANALYSIS OF ANTI-*PLASMODIUM* IMMUNE RESPONSE OF PENGUINS THAT WERE RELEASED VS PENGUINS THAT DIED DURING REHABILITATION.

1.1. Released vs Died: February 2003 to March 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		111

Note: Due to missing values, only 94 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	9.02482533	1.80496507	5.69	0.0001
Error	88	27.91764442	0.31724596		
Corrected Total	93	36.94246974			

R-Square	Coeff Var	Root MSE	Resp Mean
0.244294	85.10982	0.563246	0.661787

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	3.26061550	3.2061550	10.28	0.0019
Time	2	5.29934243	2.64947122	8.35	0.0005
Trt*time	3	0.4648670	0.23243370	0.73	0.4835

1.1.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	RESP	TIME	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.162	1	3	0.489	1	2	0.534
1	2	0.228	1	1	0.116	1	3	0.570

1	1	0.423						
1	2	0.188	1	2	0.188	1	2	0.188
1	3	0.750	1	3	0.750	1	3	0.750
1	1	0.161	1	1	0.161	1	1	0.161
1	2	0.371	1	2	0.371	1	2	0.371
1	3	.	1	3	.	1	3	.
1	1	0.092	1	1	0.092	1	1	0.092
1	2	0.618	1	2	0.618	1	2	0.618
1	3	0.841	1	3	0.841	1	3	0.841
1	1	0.059	1	1	0.059	1	1	0.059
1	2	0.821	1	2	0.821	1	2	0.821
1	3	1.089	1	3	1.089	1	3	1.089
1	1	0.003	1	1	0.003	1	1	0.003
1	2	0.331	1	2	0.331	1	2	0.331
1	3	0.927	1	3	0.927	1	3	0.927
1	1	0.420	1	1	0.420	1	1	0.420
1	2	1.429	1	2	1.429	1	2	1.429
1	3	2.318	1	3	2.318	1	3	2.318
1	1	0.293	1	1	0.293	1	1	0.293
1	2	0.575	1	2	0.575	1	2	0.575
1	3	0.649	1	3	0.649	1	3	0.649
1	1	0.195	1	1	0.195	1	1	0.195
1	2	0.585	1	2	0.585	1	2	0.585
1	3	0.113	1	3	0.113	1	3	0.113
1	1	0.465	1	1	0.465	1	1	0.465
1	2	0.723	1	2	0.723	1	2	0.723
1	3	1.076	1	3	1.076	1	3	1.076
1	1	0.708	1	1	0.708			
1	2	0.473	1	2	0.473			
1	3	2.064	1	3	2.064			
1	1	0.359	1	1	0.359			
1	2	0.168	1	2	0.168			
1	3	1.149	1	3	1.149			
1	1	0.353	1	1	0.353			
1	2	0.047	1	2	0.047			
1	3	0.607	1	3	0.607			
1	1	0.373	1	1	0.373			
1	2	0.122	1	2	0.122			
1	3	0.565	1	3	0.565			

Treatment (TRT): 1 = Released group
2 = Died group

Time: Weeks after admission

Response (RESP): ELISA titre

1.2. Released vs Died: April 2003 to May 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		156

Note: Due to missing values, only 118 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	3	1.27076738	0.42358913	2.50	0.0627
Error	114	19.27761995	0.16910193		
Corrected Total	117	20.54838733			

R-Square	Coeff Var	Root MSE	Resp Mean
0.061843	74.44724	0.411220	0.552364

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.22124013	0.22124013	1.31	0.2551
Time	2	1.04952724	0.52476362	3.10	0.0487
Trt*time	0	0.00000000			

1.2.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.466	1	2	0.289	1	3	.
1	2	0.311	1	3	0.211	1	1	0.229
1	3	0.179	1	1	0.106	1	2	0.528
1	1	0.505	1	2	0.160	1	3	.
1	2	0.266	1	3	0.202	1	1	0.119
1	3	.	1	1	0.043	1	2	.
1	1	0.619	1	2	.	1	3	.

1	1	0.115	1	1	0.669	1	1	0.322
1	2	.	1	2	0.977	1	2	0.751
1	3	.	1	3	.	1	3	0.286
1	1	0.435	1	1	0.455	1	1	1.176
1	2	.	1	2	0.859	1	2	0.807
1	3	.	1	3	0.571	1	3	.
1	1	0.660	1	1	0.683	1	1	0.500
1	2	1.165	1	2	0.585	1	2	0.489
1	3	.	1	3	.	1	3	.
1	1	0.136	1	1	0.383	1	1	0.327
1	2	0.142	1	2	1.139	1	2	0.744
1	3	0.305	1	3	.	1	3	0.298
1	1	0.070	1	1	0.870	1	1	0.624
1	2	.	1	2	.	1	2	0.707
1	3	.	1	3	0.419	1	3	0.810
1	1	0.107	1	1	0.535	1	1	0.484
1	2	.	1	2	1.022	1	2	0.502
1	3	.	1	3	.	1	3	.
1	1	1.474	1	1	0.159	1	1	0.311
1	2	0.995	1	2	0.803	1	2	0.274
1	3	.	1	3	0.605	1	3	.
1	1	0.981	1	1	0.303	1	1	0.507
1	2	0.903	1	2	0.527	1	2	.
1	3	2.922	1	3	.	1	3	.
1	1	0.719	1	1	0.792	1	1	0.740
1	2	1.218	1	2	0.724	1	2	0.438
1	3	.	1	3	0.511	1	3	.
1	1	0.649	1	1	0.359	1	1	0.685
1	2	1.972	1	2	0.791	1	2	0.832
1	3	0.098	1	3	.	1	3	0.481
1	1	0.292	1	1	0.730	1	1	0.586
1	2	0.735	1	2	0.627	1	2	1.809
1	3	.	1	3	.	1	3	0.352
1	1	0.664	1	1	0.693	1	1	0.258
1	2	1.413	1	2	0.707	1	2	0.105
1	3	.	1	3	0.300	1	3	0.705
1	1	0.186	1	1	0.913	1	1	0.242
1	2	0.867	1	2	0.764	1	2	0.176
1	3	0.277	1	3	.	1	3	0.544

1	1	0.057	1	2	0.226	1	3	0.532
1	2	0.209	1	3	0.482	2	1	0.084
1	3	0.423	1	1	0.298	2	2	.
1	1	0.066	1	2	0.208	2	3	.
1	2	0.094	1	3	0.548			
1	3	.	1	1	0.505			
1	1	0.088	1	2	0.279			

1.3. Released vs Died: June 2003 to July 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		207

Note: Due to missing values, only 190 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	4	0.11808291	0.02952073	0.21	0.9309
Error	185	25.61863036	0.13847908		
Corrected Total	189	25.73671327			

R-Square	Coeff Var	Root MSE	Resp Mean
0.004588	104.5813	0.372128	0.355826

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.00576968	0.00576968	0.04	0.8385
Time	2	0.10735306	0.05367653	0.39	0.6792
Trt*time	1	0.00496016	0.00496016	0.04	0.8501

1.3.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.777	1	3	0.208	1	2	0.168
1	2	0.784	1	1	0.107	1	3	0.148
1	3	0.267	1	2	0.288	1	1	0.163
1	1	0.094	1	3	.	1	2	0.14
1	2	0.227	1	1	0.134	1	3	0.331
1	3	0.183	1	2	0.385	1	1	0.108
1	1	0.905	1	3	0.36	1	2	0.312
1	2	0.757	1	1	0.071	1	3	0.211
1	3	0.307	1	2	0.265	1	1	0.898
1	1	2.07	1	3	0.175	1	2	0.363
1	2	.	1	1	0.095	1	3	0.135
1	3	0.717	1	2	0.311	1	1	1.648
1	1	0.998	1	3	0.152	1	2	0.39
1	2	.	1	1	0.061	1	3	0.149
1	3	0.438	1	2	0.539	1	1	0.747
1	1	0.492	1	3	0.25	1	2	0.344
1	2	0.287	1	1	0.03	1	3	0.169
1	3	.	1	2	0.279	1	1	0.405
1	1	0.258	1	3	0.215	1	2	0.406
1	2	0.422	1	1	0.199	1	3	0.195
1	3	0.192	1	2	1.467	1	1	0.425
1	1	0.268	1	3	0.359	1	2	.
1	2	0.918	1	1	0.216	1	3	.
1	3	0.245	1	2	0.555	1	1	0.267
1	1	0.453	1	3	2.513	1	2	.
1	2	0.627	1	1	0.387	1	3	.
1	3	0.329	1	2	0.474	1	1	0.267
1	1	0.288	1	3	0.404	1	2	.
1	2	1.199	1	1	0.264	1	3	.
1	3	0.271	1	2	0.447	1	1	0.609
1	1	0.025	1	3	0.26	1	2	0.154
1	2	.	1	1	0.189	1	3	0.128
1	3	.	1	2	0.324	1	1	0.627
1	1	0.129	1	3	0.197	1	2	0.164
1	2	0.131	1	1	0.257	1	3	0.163
1	3	0.136	1	2	0.664	1	1	0.676
1	1	0.181	1	3	.	1	2	.
1	2	0.971	1	1	0.123	1	3	0.106

1	1	0.645	1	3	0.309	1	2	0.312
1	2	0.176	1	1	0.219	1	3	0.5
1	3	0.165	1	2	0.104	1	1	0.157
1	1	0.171	1	3	0.167	1	2	0.238
1	2	0.435	1	1	0.251	1	3	0.39
1	3	0.168	1	2	0.119	1	1	0.7
1	1	0.154	1	3	0.185	1	2	0.149
1	2	2.371	1	1	0.121	1	3	0.259
1	3	1.894	1	2	0.071	1	1	0.559
1	1	0.109	1	3	0.197	1	2	0.549
1	2	0.156	1	1	0.083	1	3	0.422
1	3	0.157	1	2	0.142	1	1	0.216
1	1	0.23	1	3	0.287	1	2	0.134
1	2	0.245	1	1	0.107	1	3	0.273
1	3	0.366	1	2	0.256	1	1	0.134
1	1	0.255	1	3	0.316	1	2	0.161
1	2	0.264	1	1	0.067	1	3	0.698
1	3	0.245	1	2	0.117	1	1	0.03
1	1	0.206	1	3	0.246	1	2	0.205
1	2	0.174	1	1	0.164	1	3	0.208
1	3	0.32	1	2	0.344	1	1	0.046
1	1	0.328	1	3	0.51	1	2	0.31
1	2	0.208	1	1	0.091	1	3	0.878
1	3	0.286	1	2	0.144	2	1	0.333
1	1	0.181	1	3	0.522	2	2	.
1	2	0.217	1	1	0.126	2	3	.
1	3	0.232	1	2	0.239	2	1	0.485
1	1	0.144	1	3	0.222	2	2	0.38
1	2	0.128	1	1	0.127	2	3	.
1	3	0.22	1	2	0.527			
1	1	0.514	1	3	0.316			
1	2	0.098	1	1	0.137			

Treatment (TRT): 1 = Released group

2 = Died group

Time: Weeks after admission

Response (RESP): ELISA titre

1.4. Released vs Died: August 2003 to September 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		237

Note: Due to missing values, only 230 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	0.72190030	0.14438006	1.10	0.3629
Error	224	29.47934919	0.13160424		
Corrected Total	229	30.20124950			

R-Square	Coeff Var	Root MSE	Resp Mean
0.023903	100.2424	0.362773	0.361896

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.07462577	0.07462577	0.57	0.4522
Time	2	0.64451455	0.32225728	2.45	0.0887
Trt*time	2	0.00275998	0.00137999	0.01	0.9896

1.4.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.343	1	2	0.366	1	3	0.518
1	2	0.264	1	3	0.285	1	1	0.111
1	3	0.211	1	1	0.683	1	2	0.969
1	1	0.591	1	2	0.691	1	3	0.244
1	2	0.196	1	3	0.548	1	1	0.544
1	3	0.131	1	1	0.554	1	2	0.749
1	1	0.570	1	2	0.762	1	3	0.101

1	1	0.658	1	1	0.801	1	1	0.192
1	2	0.593	1	2	0.141	1	2	0.113
1	3	0.242	1	3	0.139	1	3	.
1	1	0.760	1	1	0.722	1	1	0.125
1	2	0.467	1	2	0.182	1	2	0.146
1	3	0.172	1	3	0.224	1	3	0.133
1	1	0.210	1	1	0.309	1	1	0.253
1	2	0.301	1	2	0.290	1	2	0.101
1	3	0.261	1	3	0.163	1	3	0.171
1	1	0.431	1	1	0.545	1	1	0.280
1	2	0.358	1	2	0.984	1	2	.
1	3	0.186	1	3	0.904	1	3	.
1	1	0.301	1	1	0.483	1	1	0.119
1	2	0.235	1	2	0.785	1	2	0.119
1	3	0.152	1	3	0.301	1	3	0.106
1	1	0.546	1	1	0.219	1	1	0.132
1	2	0.531	1	2	0.697	1	2	0.131
1	3	0.157	1	3	0.162	1	3	0.239
1	1	0.420	1	1	0.320	1	1	0.065
1	2	0.305	1	2	0.395	1	2	0.277
1	3	0.352	1	3	0.078	1	3	0.236
1	1	0.462	1	1	0.188	1	1	0.222
1	2	0.713	1	2	0.081	1	2	0.174
1	3	0.161	1	3	0.080	1	3	0.123
1	1	0.269	1	1	0.587	1	1	0.119
1	2	0.431	1	2	0.115	1	2	.
1	3	0.473	1	3	0.463	1	3	0.181
1	1	0.227	1	1	0.425	1	1	0.138
1	2	0.199	1	2	0.192	1	2	0.147
1	3	0.158	1	3	0.192	1	3	0.146
1	1	0.365	1	1	0.237	1	1	0.365
1	2	0.760	1	2	0.125	1	2	0.243
1	3	2.648	1	3	0.211	1	3	0.272
1	1	0.686	1	1	0.372	1	1	0.200
1	2	0.230	1	2	0.158	1	2	0.097
1	3	0.081	1	3	.	1	3	0.344
1	1	0.316	1	1	0.414	1	1	0.095
1	2	0.139	1	2	0.235	1	2	0.183
1	3	0.090	1	3	0.353			

1	3	0.311	1	1	0.281
1	1	0.257	1	2	0.243
1	2	0.335	1	3	0.987
1	3	0.188	1	1	0.209
1	1	0.123	1	2	0.248
1	2	0.162	1	3	2.165
1	3	0.212	1	1	0.124
1	1	0.109	1	2	0.270
1	2	0.171	1	3	1.133
1	3	0.161	1	1	0.074
1	1	0.184	1	2	0.191
1	2	0.249	1	3	0.152
1	3	0.550	1	1	0.171
1	1	0.082	1	2	0.226
1	2	0.164	1	3	2.249
1	3	0.268	1	1	0.164
1	1	0.084	1	2	0.155
1	2	0.092	1	3	0.880
1	3	0.535	1	1	0.121
1	1	0.109	1	2	0.304
1	2	0.123	1	3	0.628
1	3	0.290	1	1	0.140
1	1	0.069	1	2	0.198
1	2	0.191	1	3	0.764
1	3	0.264	1	1	0.153
1	1	0.149	1	2	0.229
1	2	0.154	1	3	0.253
1	3	0.391	1	1	0.265
1	1	0.129	1	2	0.318
1	2	0.180	1	3	0.342
1	3	0.212	1	1	0.105
1	1	0.121	1	2	0.068
1	2	0.118	1	3	0.889
1	3	0.431	1	1	1.607
1	2	0.299			
1	3	0.631			
1	1	0.068			
1	2	1.648			
1	3	0.322			
1	1	0.605			
1	2	0.396			
1	3	1.191			
1	1	0.419			
1	2	0.383			
1	3	0.381			
1	1	0.354			
1	2	0.252			
1	3	0.290			
1	1	0.450			
1	2	0.366			
1	3	0.344			
1	1	0.621			
1	2	0.337			
1	3	2.161			
1	1	0.346			
1	2	1.032			
1	3	0.209			
1	1	0.326			
1	2	0.311			
1	3	0.378			
2	1	0.158			
2	2	.			
2	3	.			
2	1	0.183			
2	2	0.237			
2	3	0.328			

Treatment (TRT): 1 = Released group

2 = Died group

Time: Weeks after admission

Response (RESP): ELISA titre

1.5. Released vs Died: October 2003 to November 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		195

Note: Due to missing values, only 180 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	1.0022033	0.20044607	2.08	0.0706
Error	174	16.80322533	0.09657026		
Corrected Total	179	17.80545566			

R-Square	Coeff Var	Root MSE	Resp Mean
0.056288	87.60452	0.310758	0.354728

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.0357136	0.03757136	0.39	0.5336
Time	2	0.85431812	0.42715906	4.42	0.0134
Trt*time	2	0.11034085	0.5517034	0.57	0.5658

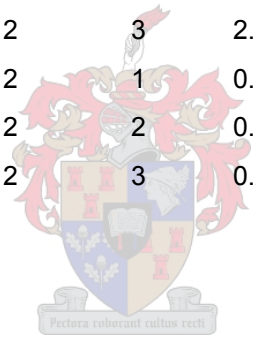
1.5.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.321	1	2	0.127	1	3	0.157
1	2	0.310	1	3	0.419	1	1	0.451
1	3	0.351	1	1	0.442	1	2	0.229
1	1	0.767	1	2	0.243	1	3	0.762
1	2	.	1	3	.	1	1	0.516
1	3	0.473	1	1	0.261	1	2	0.269
1	1	0.337	1	2	0.141	1	3	.

1	1	0.617	1	1	0.239	1	1	0.359
1	2	0.411	1	2	0.564	1	2	0.379
1	3	0.228	1	3	0.108	1	3	0.426
1	1	0.340	1	1	0.218	1	1	0.151
1	2	0.222	1	2	0.361	1	2	0.406
1	3	0.384	1	3	0.772	1	3	0.417
1	1	0.383	1	1	0.159	1	1	0.112
1	2	0.189	1	2	0.392	1	2	0.235
1	3	0.340	1	3	0.422	1	3	0.365
1	1	0.240	1	1	0.301	1	1	0.372
1	2	0.193	1	2	0.621	1	2	.
1	3	0.199	1	3	2.683	1	3	0.436
1	1	0.427	1	1	0.298	1	1	0.141
1	2	0.325	1	2	0.185	1	2	0.221
1	3	0.297	1	3	0.268	1	3	0.225
1	1	0.318	1	1	0.274	1	1	0.138
1	2	0.249	1	2	0.425	1	2	0.327
1	3	0.307	1	3	0.589	1	3	0.532
1	1	0.460	1	1	0.189	1	1	0.086
1	2	0.250	1	2	0.200	1	2	0.682
1	3	0.235	1	3	0.374	1	3	0.511
1	1	0.768	1	1	0.293	1	1	0.450
1	2	0.192	1	2	0.184	1	2	0.292
1	3	0.330	1	3	0.547	1	3	0.353
1	1	0.512	1	1	0.278	1	1	0.870
1	2	0.194	1	2	0.453	1	2	0.114
1	3	0.419	1	3	0.172	1	3	0.072
1	1	0.094	1	1	0.475	1	1	0.574
1	2	0.183	1	2	0.440	1	2	0.305
1	3	0.192	1	3	1.562	1	3	0.197
1	1	0.205	1	1	0.236	1	1	0.107
1	2	0.278	1	2	.	1	2	0.310
1	3	0.436	1	3	.	1	3	0.353
1	1	0.160	1	1	0.306	1	1	0.129
1	2	.	1	2	.	1	2	0.217
1	3	0.348	1	3	.	1	3	0.318
1	1	0.225	1	1	0.286	1	1	0.229
1	2	0.237	1	2	0.223	1	2	0.417
1	3	0.388	1	3	1.493			

1	3	0.109	1	2	0.111	2	1	0.478
1	1	0.627	1	3	0.291	2	2	0.661
1	2	0.177	2	1	0.207	2	3	0.375
1	3	0.235	2	2	0.215	2	1	0.388
1	1	0.110	2	3	0.504	2	2	0.537
1	2	0.168	2	1	0.585	2	3	0.181
1	3	0.162	2	2	.	2	1	0.123
1	1	0.251	2	3	.	2	2	0.258
1	2	0.114	2	1	0.362	2	3	0.162
1	3	0.581	2	2	.	2	1	0.291
1	1	0.301	2	3	.	2	2	0.559
1	2	0.206	2	1	0.360	2	3	0.296
1	3	0.339	2	2	0.383	2	1	0.099
1	1	0.121	2	3	0.161	2	2	0.066
1	2	0.206	2	1	0.094	2	3	0.257
1	3	0.107	2	2	0.440	2	1	0.307
1	1	0.589	2	3	2.554	2	2	.
1	2	0.253	2	1	0.126	2	3	.
1	3	0.291	2	2	0.347			
1	1	0.122	2	3	0.235			



Treatment (TRT): 1 = Released group

2 = Died group

Time: Weeks after admission

Response (RESP): ELISA titre

1.6. Released vs Died: December 2003 to January 2004.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		141

Note: Due to missing values, only 125 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	4.60165803	0.92033161	3.37	0.0069
Error	119	32.47265536	0.27287946		
Corrected Total	124	37.07431339			

R-Square	Coeff Var	Root MSE	Resp Mean
0.124120	125.3284	0.522379	0.416808

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.02192955	0.02192955	0.08	0.7773
Time	2	3.52426701	1.76213350	6.46	0.0022
Trt*time	2	1.05546147	0.52773074	1.93	0.1491

1.6.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.124	1	3	0.449	1	2	0.185
1	2	0.186	1	1	0.235	1	3	0.335
1	3	0.609	1	2	0.220	1	1	0.106
1	1	0.347	1	3	0.497	1	2	0.151
1	2	.	1	1	0.408	1	3	0.030
1	3	0.195	1	2	0.170	1	1	0.132
1	1	0.596	1	3	.	1	2	0.204
1	2	0.269	1	1	0.179	1	3	0.129
1	3	.	1	2	0.049	1	1	0.090
1	1	0.293	1	3	.	1	2	0.139
1	2	0.437	1	1	0.019	1	3	0.144
1	3	0.441	1	2	0.166	1	1	0.308
1	1	1.304	1	3	0.029	1	2	0.210
1	2	0.107	1	1	0.413	1	3	0.089
1	3	2.961	1	2	2.009	1	1	0.087
1	1	0.138	1	3	1.273	1	2	0.247
1	2	.	1	1	0.302	1	3	0.133
1	3	0.634	1	2	0.167	1	1	0.111
1	1	0.374	1	3	0.083	1	2	0.104
1	2	0.531	1	1	0.157	1	3	0.029

1	1	0.195	1	2	0.806
1	2	0.014	1	3	2.192
1	3	.	1	1	0.599
1	1	0.010	1	2	0.440
1	2	0.031	1	3	.
1	3	0.138	1	1	0.392
1	1	0.066	1	2	0.590
1	2	0.058	1	3	2.747
1	3	0.310	1	1	0.420
1	1	0.037	1	2	0.367
1	2	0.004	1	3	.
1	3	0.146	1	1	0.346
1	1	0.428	1	2	0.162
1	2	0.226	1	3	0.071
1	3	0.560	1	1	0.573
1	1	0.243	1	2	0.193
1	2	.	1	3	0.416
1	3	0.418	1	1	0.726
1	1	0.247	1	2	0.470
1	2	0.898	1	3	0.518
1	3	0.376	2	1	0.099
1	1	0.176	2	2	0.066
1	2	0.299	2	3	0.257
1	3	0.954	2	1	0.307
1	1	0.152	2	2	.
1	2	0.630	2	3	.
1	3	1.007	2	1	0.050
1	1	1.607	2	2	.
			2	3	.
			2	1	0.368
			2	2	0.059
			2	3	.
			2	1	0.491
			2	2	0.438
			2	3	2.770
			2	1	0.205
			2	2	0.414
			2	3	2.381
			2	1	0.159
			2	2	0.209
			2	3	0.493
			2	1	0.463
			2	2	0.220
			2	3	0.381
			2	1	0.090
			2	2	.
			2	3	.
			2	1	0.032
			2	2	0.220
			2	3	0.434
			2	1	0.283
			2	2	0.273
			2	3	0.347

Treatment (TRT):

1 = Released group

2 = Died group

Time: Weeks after admission

Response (RESP): ELISA titre

2. STATISTICAL ANALYSIS OF ANTI-*PLASMODIUM* IMMUNE RESPONSE OF PENGUINS THAT WERE OILED VS PENGUINS THAT WERE NOT OILED.

2.1. Oiled vs Non-Oiled: February 2003 to March 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		111

Note: Due to missing values, only 95 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	6.92724286	1.38544857	4.08	0.0022
Error	89	30.21075440	0.33944668		
Corrected Total	94	37.13799727			

R-Square	Coeff Var	Root MSE	Resp Mean
0.186527	88.57915	0.582621	0.657740

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.30093855	0.30093855	0.89	0.3490
Time	2	6.61675781	3.30837890	9.75	0.0001
Trt*time	2	0.00954651	0.00477326	0.01	0.9860

2.1.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.092	1	1	0.048	1	1	0.997
1	2	0.618	1	2	0.445	1	2	0.442
1	3	0.841	1	3	.	1	3	.
1	1	0.059	1	1	0.003	2	1	0.162
1	2	0.821	1	2	0.331	2	2	0.228
1	3	1.089	1	3	0.927	2	3	0.489

2	1	0.096	2	3	1.076
2	2	.	2	1	0.708
2	3	.	2	2	0.473
2	1	0.116	2	3	2.064
2	2	0.534	2	1	0.309
2	3	0.570	2	2	0.189
2	1	0.060	2	3	.
2	2	.	2	1	0.359
2	3	.	2	2	0.168
2	1	0.200	2	3	1.149
2	2	0.267	2	1	0.353
2	3	.	2	2	0.047
2	1	0.423	2	3	0.607
2	2	0.188	2	1	0.373
2	3	0.750	2	2	0.122
2	1	0.329	2	3	0.565
2	2	0.480	2	1	0.777
2	3	.	2	2	0.243
2	1	0.305	2	3	0.787
2	2	.	2	1	0.081
2	3	.	2	2	0.791
2	1	0.420	2	3	2.468
2	2	1.429	2	1	0.837
2	3	2.318	2	2	1.174
2	1	0.293	2	3	1.562
2	2	0.575	2	1	0.378
2	3	0.649	2	2	2.066
2	1	0.195	2	3	2.917
2	2	0.585	2	1	0.665
2	3	0.113	2	2	2.993
2	1	0.465	2	3	1.594
2	2	0.723	2	1	1.085
2	2	1.714			
2	3	0.607			
2	1	0.330			
2	2	0.227			
2	3	1.284			
2	1	1.188			
2	2	1.025			
2	3	0.420			
2	1	0.078			
2	2	0.262			
2	3	1.337			
2	1	1.694			
2	2	0.195			
2	3	.			
2	1	0.019			
2	2	.			
2	3	.			
2	1	0.361			
2	2	0.398			
2	3	0.157			
2	1	0.328			
2	2	0.548			
2	3	.			
2	1	0.355			
2	2	0.320			
2	3	.			
2	1	0.273			
2	2	0.357			
2	3	0.353			

Treatment (TRT): 1 = Oiled group

2 = Non-oiled group

Time: Weeks after admission

Response (RESP): ELISA titre

2.2. Oiled vs Non-Oiled: April 2003 to May 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		156

Note: Due to missing values, only 118 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	2.48431531	0.4968306	3.08	0.0121
Error	112	18.06363189	0.16128243		
Corrected Total	117	20.54794719			

R-Square	Coeff Var	Root MSE	Resp Mean
0.120903	72.43222	0.401600	0.554449

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	1.60140382	1.60140382	9.93	0.0021
Time	2	0.82665879	0.41332939	2.56	0.0816
Trt*time	2	0.05625270	0.02812635	0.17	0.8402

2.2.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.619	1	1	0.719	1	1	0.664
1	2	0.289	1	2	1.218	1	2	1.413
1	3	0.211	1	3	.	1	3	.
1	1	1.474	1	1	0.649	1	1	0.186
1	2	0.995	1	2	1.972	1	2	0.867
1	3	.	1	3	0.098	1	3	0.277
1	1	0.981	1	1	0.292	1	1	0.669
1	2	0.903	1	2	0.735	1	2	0.977
1	3	2.922	1	3	.	1	3	.

1	1	0.455	1	1	1.176	1	1	0.298
1	2	0.859	1	2	0.807	1	2	0.208
1	3	0.571	1	3	.	1	3	0.548
1	1	0.683	1	1	0.5	2	1	0.466
1	2	0.585	1	2	0.489	2	2	0.311
1	3	.	1	3	.	2	3	0.179
1	1	0.383	1	1	0.627	2	1	0.505
1	2	1.139	1	2	0.744	2	2	0.266
1	3	.	1	3	0.298	2	3	.
1	1	0.87	1	1	0.624	2	1	0.106
1	2	.	1	2	0.707	2	2	0.106
1	3	0.419	1	3	0.81	2	3	0.202
1	1	0.535	1	1	0.484	2	1	0.043
1	2	1.022	1	2	0.502	2	2	.
1	3	.	1	3	.	2	3	.
1	1	0.159	1	1	0.311	2	1	0.229
1	2	0.803	1	2	0.274	2	2	0.528
1	3	0.605	1	3	.	2	3	.
1	1	0.303	1	1	0.507	2	1	0.119
1	2	0.527	1	2	.	2	2	.
1	3	.	1	3	.	2	3	.
1	1	0.792	1	1	0.74	2	1	0.115
1	2	0.724	1	2	0.438	2	2	.
1	3	0.511	1	3	.	2	3	.
1	1	0.359	1	1	0.685	2	1	0.435
1	2	0.791	1	2	0.832	2	2	.
1	3	.	1	3	0.481	2	3	.
1	1	0.73	1	1	0.258	2	1	0.66
1	2	0.627	1	2	0.105	2	2	1.165
1	3	.	1	3	0.705	2	3	.
1	1	0.693	1	1	0.242	2	1	0.136
1	2	0.707	1	2	0.176	2	2	0.142
1	3	0.3	1	3	0.544	2	3	0.305
1	1	0.913	1	1	0.057	2	1	0.084
1	2	0.764	1	2	0.209	2	2	.
1	3	.	1	3	0.423	2	3	.
1	1	0.322	1	1	0.066	2	1	0.07
1	2	0.751	1	2	0.094	2	2	.
1	3	0.286	1	3	.			

2	3	.	2	2	1.809	2	1	0.505
2	1	0.107	2	3	0.352	2	2	0.279
2	2	.	2	1	0.088	2	3	0.532
2	3	.	2	2	0.226			
2	1	0.586	2	3	0.482			

Treatment (TRT): 1 = Oiled group

2 = Non-oiled group

Time: Weeks after admission

Response (RESP): ELISA titre

2.3. Oiled vs Non-Oiled: June 2003 to July 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		210

Note: Due to missing values, only 193 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	0.31343738	0.06268748	0.46	0.8065
Error	187	25.55216560	0.13664260		
Corrected Total	192	25.86560298			

R-Square	Coeff Var	Root MSE	Resp Mean
0.12118	104.7388	0.369652	0.352927

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.00126020	0.00126020	0.01	0.9236
Time	2	0.11647904	0.05823952	0.43	0.6536
Trt*time	2	0.19569814	0.09784907	0.72	0.4900

2.3.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.777	1	3	0.175	1	2	0.390
1	2	0.784	1	1	0.095	1	3	0.149
1	3	0.267	1	2	0.311	1	1	0.267
1	1	0.905	1	3	0.152	1	2	.
1	2	0.757	1	1	0.061	1	3	.
1	3	0.307	1	2	0.539	1	1	0.627
1	1	2.070	1	3	0.205	1	2	0.164
1	2	.	1	1	0.030	1	3	0.163
1	3	0.717	1	2	0.279	1	1	0.676
1	1	0.998	1	3	0.215	1	2	.
1	2	.	1	1	0.199	1	3	0.106
1	3	0.438	1	2	1.467	1	1	0.645
1	1	0.492	1	3	0.359	1	2	0.176
1	2	0.257	1	1	0.216	1	3	0.165
1	3	.	1	2	0.555	1	1	0.171
1	1	0.258	1	3	2.513	1	2	0.435
1	2	0.422	1	1	0.387	1	3	0.168
1	3	0.192	1	2	0.474	1	1	0.109
1	1	0.268	1	3	0.404	1	2	0.156
1	2	0.918	1	1	0.264	1	3	0.157
1	3	0.245	1	2	0.447	1	1	0.230
1	1	0.453	1	3	0.260	1	2	0.245
1	2	0.627	1	1	0.189	1	3	0.366
1	3	0.329	1	2	0.324	1	1	0.255
1	1	0.288	1	3	0.197	1	2	0.264
1	2	1.199	1	1	0.257	1	3	0.245
1	3	0.271	1	2	0.664	1	1	0.206
1	1	0.129	1	3	.	1	2	0.174
1	2	0.131	1	1	0.123	1	3	0.320
1	3	0.136	1	2	0.168	1	1	0.328
1	1	0.181	1	3	0.148	1	2	0.208
1	2	0.971	1	1	0.108	1	3	0.286
1	3	0.208	1	2	0.312	1	1	0.181
1	1	0.107	1	3	0.211	1	2	0.217
1	2	0.288	1	1	0.898	1	3	0.232
1	3	.	1	2	0.363	1	1	0.144
1	1	0.071	1	3	0.135	1	2	0.128
1	2	0.265	1	1	1.648	1	3	0.220

1	1	0.251	1	1	0.700
1	2	0.119	1	2	0.149
1	3	0.185	1	3	0.259
1	1	0.121	1	1	0.559
1	2	0.071	1	2	0.546
1	3	0.197	1	3	0.422
1	1	0.083	1	1	0.216
1	2	0.142	1	2	0.134
1	3	0.287	1	3	0.273
1	1	0.107	1	1	0.134
1	2	0.256	1	2	0.161
1	3	0.316	1	3	0.698
1	1	0.067	1	1	0.485
1	2	0.117	1	2	0.380
1	3	0.246	1	3	.
1	1	0.164	2	1	0.036
1	2	0.344	2	2	0.299
1	3	0.510	2	3	0.253
1	1	0.091	2	1	0.094
1	2	0.144	2	2	0.227
1	3	0.522	2	3	0.183
1	1	0.126	2	1	0.025
1	2	0.239	2	2	.
1	3	0.222	2	3	.
1	1	0.127	2	1	0.134
1	2	0.527	2	2	0.385
1	3	0.316	2	3	0.360
1	1	0.137	2	1	0.163
1	2	0.312	2	2	0.140
1	3	0.500	2	3	0.331
1	1	0.157	2	1	0.747
1	2	0.238	2	2	0.344
1	3	0.390	2	3	0.169
			2	1	0.405
			2	2	0.406
			2	3	0.195
			2	1	0.425
			2	2	.
			2	3	.
			2	1	0.267
			2	2	.
			2	3	.
			2	1	0.607
			2	2	0.154
			2	3	0.128
			2	1	0.154
			2	2	2.371
			2	3	1.894
			2	1	0.333
			2	2	.
			2	3	.
			2	1	0.514
			2	2	0.098
			2	3	0.309
			2	1	0.219
			2	2	0.104
			2	3	0.167
			2	1	0.030
			2	2	0.205
			2	3	0.208
			2	1	0.046
			2	2	0.310
			2	3	0.878

Treatment (TRT): 1 = Oiled group

2 = Non-oiled group

Time: Weeks after admission

Response (RESP): ELISA titre

2.4. Oiled vs Non-Oiled: August 2003 to September 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		216

Note: Due to missing values, only 209 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	0.96926201	0.19385240	1.59	0.1637
Error	203	24.71429105	0.12174528		
Corrected Total	208	25.68355306			

R-Square	Coeff Var	Root MSE	Resp Mean
0.037739	100.7715	0.348920	0.346249

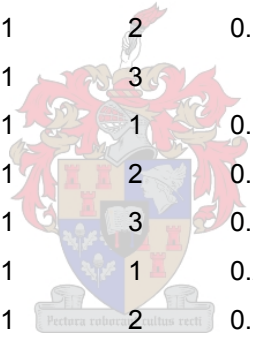
Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.02914320	0.02914320	0.24	0.6252
Time	2	0.42989916	0.21494958	1.77	0.1737
Trt*time	2	0.51021966	0.25510983	2.10	0.1257

2.4.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.343	1	3	0.285	1	2	0.969
1	2	0.264	1	1	0.683	1	3	0.244
1	3	0.211	1	2	0.691	1	1	0.658
1	1	0.591	1	3	0.548	1	2	0.593
1	2	0.196	1	1	0.554	1	3	0.242
1	3	0.131	1	2	0.762	1	1	0.210
1	1	0.570	1	3	0.518	1	2	0.301
1	2	0.366	1	1	0.111	1	3	0.261

1	1	0.301	1	1	0.188	1	1	0.183
1	2	0.235	1	2	0.081	1	2	0.237
1	3	0.152	1	3	0.080	1	3	0.328
1	1	0.546	1	1	0.587	1	1	0.138
1	2	0.531	1	2	0.115	1	2	0.147
1	3	0.157	1	3	0.463	1	3	0.146
1	1	0.420	1	1	0.237	1	1	0.365
1	2	0.305	1	2	0.125	1	2	0.243
1	3	0.352	1	3	0.211	1	3	0.272
1	1	0.462	1	1	0.372	1	1	0.200
1	2	0.713	1	2	0.158	1	2	0.097
1	3	0.161	1	3	.	1	3	0.344
1	1	0.269	1	1	0.414	1	1	0.095
1	2	0.431	1	2	0.235	1	2	0.183
1	3	0.473	1	3	0.353	1	3	0.311
1	1	0.227	1	1	0.192	1	1	0.257
1	2	0.199	1	2	0.113	1	2	0.335
1	3	0.158	1	3	.	1	3	0.188
1	1	0.365	1	1	0.125	1	1	0.123
1	2	0.760	1	2	0.146	1	2	0.162
1	3	2.648	1	3	0.133	1	3	0.212
1	1	0.686	1	1	0.235	1	1	0.109
1	2	0.230	1	2	0.101	1	2	0.171
1	3	0.081	1	3	0.171	1	3	0.161
1	1	0.319	1	1	0.119	1	1	0.184
1	2	0.139	1	2	0.119	1	2	0.249
1	3	0.090	1	3	0.106	1	3	0.550
1	1	0.801	1	1	0.132	1	1	0.082
1	2	0.141	1	2	0.131	1	2	0.164
1	3	0.139	1	3	0.239	1	3	0.268
1	1	0.722	1	1	0.065	1	1	0.084
1	2	0.182	1	2	0.277	1	2	0.092
1	3	0.224	1	3	0.236	1	3	0.535
1	1	0.306	1	1	0.222	1	1	0.109
1	2	0.290	1	2	0.174	1	2	0.123
1	3	0.163	1	3	0.123	1	3	0.290
1	1	0.545	1	1	0.119	1	1	0.069
1	2	0.984	1	2	.	1	2	0.191
1	3	0.904	1	3	0.181			



1	3	0.264	1	2	0.155	2	1	0.431
1	1	0.149	1	3	0.880	2	2	0.658
1	2	0.154	1	1	0.121	2	3	0.186
1	3	0.391	1	2	0.304	2	1	0.483
1	1	0.129	1	3	0.628	2	2	0.785
1	2	0.180	1	1	0.140	2	3	0.301
1	3	0.212	1	2	0.198	2	1	0.219
1	1	0.121	1	3	0.764	2	2	0.697
1	2	0.118	1	1	0.153	2	3	0.162
1	3	0.431	1	2	0.229	2	1	0.320
1	1	0.281	1	3	0.253	2	2	0.395
1	2	0.243	1	1	0.265	2	3	0.078
1	3	0.987	1	2	0.318	2	1	0.425
1	1	0.209	1	3	0.342	2	2	0.192
1	2	0.248	1	1	1.607	2	3	0.192
1	3	2.165	1	2	0.299	2	1	0.158
1	1	0.124	1	3	0.631	2	2	.
1	2	0.270	1	1	0.068	2	3	.
1	3	1.133	1	2	1.648	2	1	0.280
1	1	0.074	1	3	0.322	2	2	.
1	2	0.191	2	1	0.544	2	3	.
1	3	0.152	2	2	0.749	2	1	0.105
1	1	0.171	2	3	0.101	2	2	0.068
1	2	0.226	2	1	0.760	2	3	0.889
1	3	2.249	2	2	0.467			
1	1	0.164	2	3	0.172			

Treatment (TRT): 1 = Oiled group

2 = Non-oiled group

Time: Weeks after admission

Response (RESP): ELISA titre

2.5. Oiled vs Non-Oiled: October 2003 to November 2003.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3

Number of observations	213

Note: Due to missing values, only 196 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	1.33537862	0.26707572	2.77	0.0194
Error	190	18.33135516	0.09648082		
Corrected Total	195	19.66673378			

R-Square	Coeff Var	Root MSE	Resp Mean
0.067900	86.60313	0.310614	0.358663

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.20265116	0.20265116	2.10	0.1489
Time	2	0.73553141	0.36776570	3.81	0.0238
Trt*time	2	0.39719605	0.19859802	2.06	0.1305

2.5.1. Input Data:

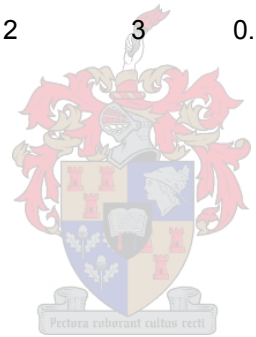
The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.605	1	2	1.032	1	3	0.157
1	2	0.396	1	3	0.209	1	1	0.451
1	3	1.191	1	1	0.326	1	2	0.229
1	1	0.419	1	2	0.311	1	3	0.792
1	2	0.383	1	3	0.378	1	1	0.516
1	3	0.381	1	1	0.767	1	2	0.269
1	1	0.354	1	2	.	1	3	.
1	2	0.252	1	3	0.473	1	1	0.617
1	3	0.290	1	1	0.337	1	2	0.411
1	1	0.450	1	2	0.127	1	3	0.228
1	2	0.366	1	3	0.419	1	1	0.340
1	3	0.344	1	1	0.442	1	2	0.222
1	1	0.321	1	2	0.243	1	3	0.384
1	2	0.337	1	3	.	1	1	0.383
1	3	2.161	1	1	0.261	1	2	0.189
1	1	0.346	1	2	0.141	1	3	0.340

1	1	0.240	1	1	0.239	1	1	0.126
1	2	0.193	1	2	0.564	1	2	0.347
1	3	0.199	1	3	0.108	1	3	0.235
1	1	0.427	1	1	0.159	1	1	0.086
1	2	0.325	1	2	0.392	1	2	0.682
1	3	0.297	1	3	0.422	1	3	0.511
1	1	0.318	1	1	0.301	1	1	0.107
1	2	0.249	1	2	0.621	1	2	0.310
1	3	0.307	1	3	2.683	1	3	0.353
1	1	0.460	1	1	0.298	1	1	0.126
1	2	0.250	1	2	0.185	1	2	0.217
1	3	0.235	1	3	0.268	1	3	0.318
1	1	0.768	1	1	0.186	1	1	0.122
1	2	0.192	1	2	0.200	1	2	0.111
1	3	0.330	1	3	0.374	1	3	0.291
1	1	0.512	1	1	0.278	2	1	0.321
1	2	0.194	1	2	0.453	2	2	0.310
1	3	0.419	1	3	0.172	2	3	0.351
1	1	0.094	1	1	0.359	2	1	0.218
1	2	0.183	1	2	0.379	2	2	0.361
1	3	0.192	1	3	0.426	2	3	0.772
1	1	0.205	1	1	0.151	2	1	0.274
1	2	0.278	1	2	0.406	2	2	0.425
1	3	0.436	1	3	0.417	2	3	0.589
1	1	0.160	1	1	0.094	2	1	0.293
1	2	.	1	2	0.440	2	2	0.184
1	3	0.348	1	3	2.554	2	3	0.547
1	1	0.207	1	1	0.112	2	1	0.360
1	2	0.215	1	2	0.235	2	2	0.383
1	3	0.504	1	3	0.365	2	3	0.161
1	1	0.585	1	1	0.372	2	1	0.236
1	2	.	1	2	.	2	2	.
1	3	.	1	3	0.436	2	3	.
1	1	0.362	1	1	0.141	2	1	0.306
1	2	.	1	2	0.221	2	2	.
1	3	.	1	3	0.225	2	3	.
1	1	0.225	1	1	0.138	2	1	0.478
1	2	0.237	1	2	0.327	2	2	0.661
1	3	0.388	1	3	0.532			

2	3	0.375	2	2	0.177	2	1	0.589
2	1	0.450	2	3	0.235	2	2	0.253
2	2	0.292	2	1	0.110	2	3	0.291
2	3	0.353	2	2	0.168	2	1	0.291
2	1	0.388	2	3	0.162	2	2	0.559
2	2	0.537	2	1	0.251	2	3	0.296
2	3	0.181	2	2	0.114	2	1	0.099
2	1	0.870	2	3	0.581	2	2	0.066
2	2	0.114	2	1	0.301	2	3	0.257
2	3	0.072	2	2	0.206	2	1	0.307
2	1	0.574	2	3	0.339	2	2	.
2	2	0.305	2	1	0.121	2	3	.
2	3	0.197	2	2	0.206	2	1	0.050
2	1	0.229	2	3	0.107	2	2	.
2	2	0.417	2	1	0.123	2	3	.
2	3	0.109	2	2	0.258			
2	1	0.627	2	3	0.162			

Treatment (TRT): 1 = Oiled group
2 = Non-oiled group
Time: Weeks after admission
Response (RESP): ELISA titre



2.6. Oiled vs Non-Oiled: December 2003 to January 2004.

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 2
time	3	1 2 3
Number of observations		105

Note: Due to missing values, only 94 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	2.72073337	0.54414667	2.08	0.0754
Error	88	23.02678956	0.26166806		
Corrected Total	93	25.74752294			

R-Square	Coeff Var	Root MSE	Resp Mean
0.105670	128.8847	0.511535	0.396894

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.37123227	0.37123227	1.42	0.2368
Time	2	2.10669052	1.05334526	4.03	0.0212
Trt*time	2	0.24281058	0.12140529	0.46	0.6303

2.6.1. Input Data:

The input data on each page is arranged in three columns (each containing trt, time and resp.) from top to bottom and from left to right.

TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.124	1	2	2.009	1	3	0.029
1	2	0.186	1	3	1.273	1	1	0.010
1	3	0.609	1	1	0.302	1	2	0.031
1	1	0.596	1	2	0.167	1	3	0.138
1	2	0.269	1	3	0.083	1	1	0.066
1	3	.	1	1	0.159	1	2	0.058
1	1	0.491	1	2	0.209	1	3	0.310
1	2	0.438	1	3	0.493	1	1	0.037
1	3	2.770	1	1	0.157	1	2	0.004
1	1	0.205	1	2	0.185	1	3	0.146
1	2	0.414	1	3	0.335	1	1	0.090
1	3	2.381	1	1	0.106	1	2	.
1	1	0.293	1	2	0.151	1	3	.
1	2	0.437	1	3	0.030	1	1	0.032
1	3	0.441	1	1	0.132	1	2	0.220
1	1	0.235	1	2	0.204	1	3	0.434
1	2	0.220	1	3	0.129	1	1	0.247
1	3	0.497	1	1	0.090	1	2	0.898
1	1	0.408	1	2	0.139	1	3	0.376
1	2	0.170	1	3	0.144	1	1	0.152
1	3	.	1	1	0.087	1	2	0.630
1	1	0.179	1	2	0.247	1	3	1.007
1	2	0.049	1	3	0.133	2	1	0.347
1	3	.	1	1	0.111	2	2	.
1	1	0.413	1	2	0.104	2	3	0.195

2	1	1.304	2	3	0.252	2	2	0.299
2	2	0.107	2	1	0.019	2	3	0.954
2	3	2.961	2	2	0.166	2	1	0.599
2	1	0.138	2	3	0.029	2	2	0.440
2	2	.	2	1	0.463	2	3	.
2	3	0.634	2	2	0.220	2	1	0.420
2	1	0.374	2	3	0.381	2	2	0.367
2	2	0.531	2	1	0.243	2	3	.
2	3	0.449	2	2	.			
2	1	0.903	2	3	0.418			
2	2	.	2	1	0.176			

Treatment (TRT): 1 = Oiled group

2 = Non-oiled group

Time: Weeks after admission

Response (RESP): ELISA titre

3. STATISTICAL ANALYSIS OF ANTI-*PLASMODIUM* IMMUNE RESPONSE OF PENGUINS IN BREEDING COLONIES.

3.1. Penguins from Breeding colonies: Robben Island, Betty's Bay and Dassen Island

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	3	1 2 3
time	3	1 2 3
Number of observations		154

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	5	0.04965404	0.00993081	1.23	0.2987
Error	148	1.19662726	0.00808532		
Corrected Total	153	1.24628131			

R-Square	Coeff Var	Root MSE	Resp Mean
0.039842	73.07932	0.089918	0.123042

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	2	0.00599730	0.00299865	0.37	0.6908
Time	2	0.03443014	0.01721507	2.13	0.1226
Trt*time	1	0.00922660	0.00922660	1.14	0.2871

3.1.1. Input Data:

The input data on each page is arranged in three columns (each containing trt and resp.) from top to bottom and from left to right.

LOC	TRT	RESP	LOC	TRT	RESP	LOC	TRT	RESP
1	1	0.209	1	1	0.090	1	1	0.158
1	1	0.133	1	1	0.100	1	1	0.068
1	1	0.146	1	1	0.047	1	2	0.082
1	1	0.203	1	1	0.001	1	2	0.047
1	1	0.180	1	1	0.173	1	2	0.044
1	1	0.104	1	1	0.081	1	2	0.103
1	1	0.111	1	1	0.127	1	2	0.097
1	1	0.258	1	1	0.110	2	1	0.088
1	1	0.119	1	1	0.256	2	1	0.104
1	1	0.185	1	1	0.123	2	1	0.064
1	1	0.032	1	1	0.070	2	1	0.121
1	1	0.182	1	1	0.108	2	1	0.054
1	1	0.097	1	1	0.080	2	1	0.137
1	1	0.141	1	1	0.126	2	1	0.047
1	1	0.125	1	1	0.077	2	1	0.087
1	1	0.144	1	1	0.068	2	1	0.084
1	1	0.039	1	1	0.095	2	1	0.042
1	1	0.044	1	1	0.073	2	1	0.034
1	1	0.103	1	1	0.162	2	1	0.012
1	1	0.056	1	1	0.086	2	1	0.049
1	1	0.295	1	1	0.163	2	1	0.027
1	1	0.043	1	1	0.075	2	1	0.052

2	1	0.105	2	2	0.057	3	1	0.026
2	1	0.155	2	2	0.150	3	1	0.124
2	1	0.269	2	2	0.184	3	1	0.334
2	1	0.120	2	2	0.110	3	1	0.122
2	1	0.126	2	3	0.130	3	1	0.191
2	1	0.135	2	3	0.275	3	1	0.207
2	1	0.093	2	3	0.173	3	1	0.208
2	1	0.059	2	3	0.278	3	1	0.143
2	1	0.067	3	1	0.101	3	1	0.166
2	1	0.134	3	1	0.351	3	1	0.086
2	1	0.087	3	1	0.126	3	1	0.101
2	1	0.041	3	1	0.084	3	1	0.143
2	1	0.896	3	1	0.188	3	1	0.062
2	1	0.074	3	1	0.128	3	1	0.168
2	1	0.081	3	1	0.126	3	1	0.155
2	2	0.078	3	1	0.106	3	1	0.086
2	2	0.087	3	1	0.065	3	1	0.115
2	2	0.159	3	1	0.098	3	1	0.086
2	2	0.078	3	1	0.184	3	1	0.096
2	2	0.081	3	1	0.094	3	1	0.107
2	2	0.194	3	1	0.092	3	1	0.099
2	2	0.042	3	1	0.225	3	1	0.234
2	2	0.057	3	1	0.092	3	1	0.065
2	2	0.092	3	1	0.138	3	1	0.030
2	2	0.041	3	1	0.129	3	1	0.191
2	2	0.195	3	1	0.098	3	1	0.085
2	2	0.205	3	1	0.241	3	1	0.043
2	2	0.161	3	1	0.092	3	1	0.099
2	2	0.262	3	1	0.103	3	1	0.067

3 1 0.083

Locality (LOC) = Breeding colonies: 1 = Betty's Bay
2 = Dassen Island
3 = Robben Island

Treatment (TRT) = Penguin's age: 1 = Adult
2 = Juvenile
3 = Chick

Response (RESP) = ELISA titre value

3.2. Penguins from Dassen Island: Adults vs Chicks:

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	1 3
Number of observations		34

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	1	0.03479394	0.03479394	1.55	0.2227
Error	32	0.71986283	0.02249571		
Corrected Total	33	0.75465677			

R-Square	Coeff Var	Root MSE	Resp Mean
0.046106	118.6669	0.149986	0.126392

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.03479394	0.03479694	1.55	0.2227

3.1.1. Input Data:

The input data on each page is arranged in three columns (each containing trt and resp.) from top to bottom and from left to right.

TRT	RESP	TRT	RESP	TRT	RESP
1	0.088	1	0.054	1	0.084
1	0.104	1	0.137	1	0.042
1	0.064	1	0.047	1	0.034
1	0.121	1	0.087	1	0.012

1	0.049	1	0.135	1	0.074
1	0.027	1	0.093	1	0.081
1	0.052	1	0.059	3	0.130
1	0.105	1	0.067	3	0.275
1	0.155	1	0.134	3	0.173
1	0.269	1	0.087	3	0.278
1	0.120	1	0.041		
1	0.126	1	0.896		

Treatment (TRT): 1 = Adults
3 = Chicks
Response (RESP): ELISA titre

3.3. Penguins from Dassen Island : Juveniles vs Chicks

The SAS system

The General Linear Model Procedure

Class Level Information		
Class	Levels	Values
trt	2	2 3
Number of observations		22

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	1	0.02646547	0.02646547	5.86	0.0251
Error	20	0.09034790	0.00451740		
Corrected Total	21	0.11681337			

R-Square	Coeff Var	Root MSE	Resp Mean
0.226562	47.86322	0.067212	0.140424

Source	DF	Type I SS	Mean Square	F Value	Pr>F
Trt	1	0.02646547	0.02646547	5.86	0.0251

3.2.1. Input Data:

The input data on each page is arranged in three columns (each containing trt and resp.) from top to bottom and from left to right.

TRT	RESP	TRT	RESP	TRT	RESP
2	0.078	2	0.092	2	0.184
2	0.087	2	0.041	2	0.110
2	0.159	2	0.195	3	0.130
2	0.078	2	0.205	3	0.275
2	0.081	2	0.161	3	0.173
2	0.194	2	0.262	3	0.278
2	0.042	2	0.057		
2	0.057	2	0.150		

Treatment (TRT): 2 = Juveniles

3 = Chicks

Response (RESP): ELISA titre